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Title: **Transcriptional Regulation of *Rhizobium meliloti* Nitrogen Fixation Genes**

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The transcriptional promoter sequences for the *Rhizobium meliloti* nitrogen fixation genes *nifA* and *nifB* were cloned to a β-galactosidase gene fusion plasmid vector and transferred by homologous recombination to a specialized transducing phage. The promoter fusions were then transduced to a recombination deficient strain of *Escherichia coli* as single-copy lysogens and analysed under defined aerobic and anaerobic conditions. The lysogenic strains contained plasmids encoding either of two transcriptional activator proteins, NifA or FixJ, produced from a constitutive plasmid promoter. The expression of the *nifA* and the *nifB* promoters was found to be sensitively regulated by the carbon source used for anaerobic fermentation or anaerobic respiration, the redox potential of the terminal electron acceptor used for anaerobic respiration, and the growth phase of anaerobic cultures. The repression of *nif* promoter expression by oxygen respiration

was specifically compared to anaerobic respiration of alternative electron acceptors. Both *nifA* and *nifB* promoter expression decreased exponentially as the reduction potential of the terminal respiration reaction increased. The repressive effect of oxygen appears to be due soley to the exponential relationship between *nif* promoter expression and the redox potential of oxygen respiration. In addition to separate fusions of the *nifA* and *nifB* promoters to β-galactosidase, a single-copy fusion of the entire *nifA-nifB* region was constructed. In this construct, plasmid-encoded FixJ protein stimulated the expression of a chromosomal *nifA* gene to produce the NifA protein, which then stimulated the expression of the *nifB* promoter. This strain produced 20-fold lower activity than a strain in which *nifB* promoter expression was stimulated by plasmid-encoded NifA protein. Finally, the *nifA* locus was found to contain a transcriptionally active element, oriented opposite to the *nifA* promoter.

Transcriptional Regulation of *Rhizobium meliloti* Nitrogen Fixation Genes

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To my surprise, my mothers endless childhood refrain, "Look it up", has proven to be wise counsel. No scientist can aspire to a higher goal than the humility to "Look it up", except perhaps to remember and appreciate the love of his parents. Thank you, Mom. This ten word declarative sentence acknowledges my debt to Dad. (count 'em)

Finally, I thank my wife Laura for her love, devotion, and patience.

I have served this canon seven years or more,
yet am no nearer science than before,
And all that I ever had I've lost thereby,
and so, God knows, have many more than I.

Geoffrey Chaucer
The Canon's Yeomans Tale from The Canterbury Tales.
c. 1390

Six hundred years later, and nothing has changed.

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LITERATURE REVIEW

The biochemical reduction of di-nitrogen gas to ammonia is commonly referred to as "nitrogen fixation". This process is exclusive to prokaryotic organisms, but occurs in many species within diverse families of bacteria. Photosynthetic "blue-green algae" (Cyanobacteriaceae), photosynthetic heterotrophs (Chromatiaceae and Chlorobiaceae), facultative anaerobes (Enterobacteriaceae), obligate aerobes (Azotobacteriaceae), and even members of the Archaebacteria contain species capable of nitrogen fixation (Postgate 1982). A family of obligate aerobic bacteria, the Rhizobiaceae, form symbiotic associations with leguminous plants and are taxonomically defined by their ablility to fix nitrogen in association with a particular plant species.

The interaction between the Rhizobia and the plant host is complex. The bacteria must first recognize and infect the root hairs of the specific host plant. Following infection, the bacteria migrate to the cytoplasm of root cells and differentiate into pleiomorphic "bacteroids", in which nitrogen fixation takes place. The bacteroids are enclosed by a plant derived membrane containing a hemoglobin-like protein (leghemoglobin) that limits the concentration of oxygen within the peribacteroid space. Although Rhizobia are considered to be obligate aerobes (but see Kiss et al. 1979), they exist within the peribacteroid membrane in a state of very low oxygen tension.

The low oxygen tension is required to protect the nitrogenase enzyme from irreversible inhibition by oxygen. The susceptibility of nitrogenase to oxygen therefore creates a paradox. Oxygen is (presumably) required for bacteroid metabolism as the terminal electron acceptor of electron transport, but is also an irreversible inhibitor of the nitrogenase enzyme that requires the energy derived from electron transport to oxygen (for a review of the biology of nitrogen fixation, see Postgate 1982).

The genetics of nitrogen fixation is as complex as the *Rhizobium* cell biology of host recognition, infection, and differentiation into bacteroids. The genes for essential proteins of the nitrogen fixation process are referred to as *nif* genes, and the complete nucleotide sequence of all known *nif* genes in *Klebsiella pneumoniae* has recently been published (Arnold *et al.* 1988). In Rhizobia, other genetic loci have been described that have a *nif* phenotype, but do not correspond to any of the known *nif* genes in *K. pneumoniae*; these are designated as *fix* genes.

The regulation of the *nifA* and *nifB* genes of *Rhizobium meliloti*, the bacteria symbiotic for alfalfa, is the subject of this thesis. The following literature review is primarily concerned with the regulation of nitrogen fixation gene expression in *R. meliloti*, but the salient features of *nif* gene regulation in other organisms will be presented as necessary.

Regulation of nitrogen assimilation versus nitrogen fixation: *Klebsiella pneumoniae* and *Rhizobium meliloti*.

The regulation of nitrogen fixation is intimately involved with the regulation of nitrogen assimilation. Ammonia is the preferred source of nitrogen for Enterobacteria (Reitzer and Magasanik 1987). Ammonia, the primary product of the nitrogenase enzyme, controls the expression of the *nifA* gene in *Klebsiella pneumoniae*, which in turn regulates the synthesis of all nitrogen fixation operons (reviewed in Gussin *et al.* 1986). The regulation of *nifA* in *K. pneumoniae* is mediated through the action of NtrC, the transcriptional regulator of nitrogen assimilation operons in Enterobacteria, most importantly the glutamine synthetase enzyme operon (Reitzer and Magasanik 1987; and see below). In *Rhodobacter capsulatus*, an organism capable of both nitrogen fixation and carbon fixation (photosynthesis), the situation is similar; *ntrC* strains are phenotypically *nif*$^-$ (Jones and Haselkorn 1989).

In contrast to *Klebsiella pneumoniae*, *R. meliloti ntrC* mutants are capable of nitrogen fixation and do not exhibit the same strict auxotrophic requirement for glutamine as Enterobacteria (but the effect may vary depending on the minimal media used for growth; see Szeto *et al.*1987). *R. meliloti* has three separate genes for glutamine synthetase (*glnA*), the enzyme responsible for assimilation of ammonia into glutamate; however, none of the three *glnA* genes are required for nitrogen fixation (Szeto *et al.* 1987). On first inspection this seems incongruous; if *R. meliloti* has no glutamine synthetase to incorporate some of the ammonia made by nitrogen fixation, what is the source of nitrogen for the bacteroid metabolism? The

answer may be that *R. meliloti* bacteroids are supplied with a source of fixed nitrogen from the plant, a theory originally championed by Kahn (Kahn *et al.* 1985). A second incongruous finding is that in nitrogen starved free-living cells of *R. meliloti*, the promoters for the nitrogen fixation operon *nifHDK*, the *fixABCX* operon, and the *nifB* gene are activated (Sundaresan *et al.* 1983a). Similarly, when the *nifHDK* promoter is fused to β-galactosidase in *Escherichia coli*, activation is dependent on the *E. coli* NtrC protein (Sundaresan *et al.* 1983b). If nitrogen (ammonia) limitation will stimulate the expression of *R. meliloti* nitrogen fixation genes in free-living cells (also by NtrC protein, see below), why not also in the symbiotic cells? Three possibilities are: *(i)* the bacteroid cells are not nitrogen limited (as Kahn proposes), *(ii)* the NtrC activation of *nif* genes is only physiologically relevant to non-symbiotic Rhizobia, or *(iii)* the activation by NtrC is a laboratory artifact. As regards *(ii)*, Rhizobia may yet be found to fix nitrogen *ex planta* (but this has never been demonstrated) when limited for nitrogen, in which case the demonstrated NtrC activation of *nif* genes could be important. However, the present evidence supports the conclusion that NtrC-mediated activation is not an important control of *nif* gene expression in *R. meliloti* bacteroids.

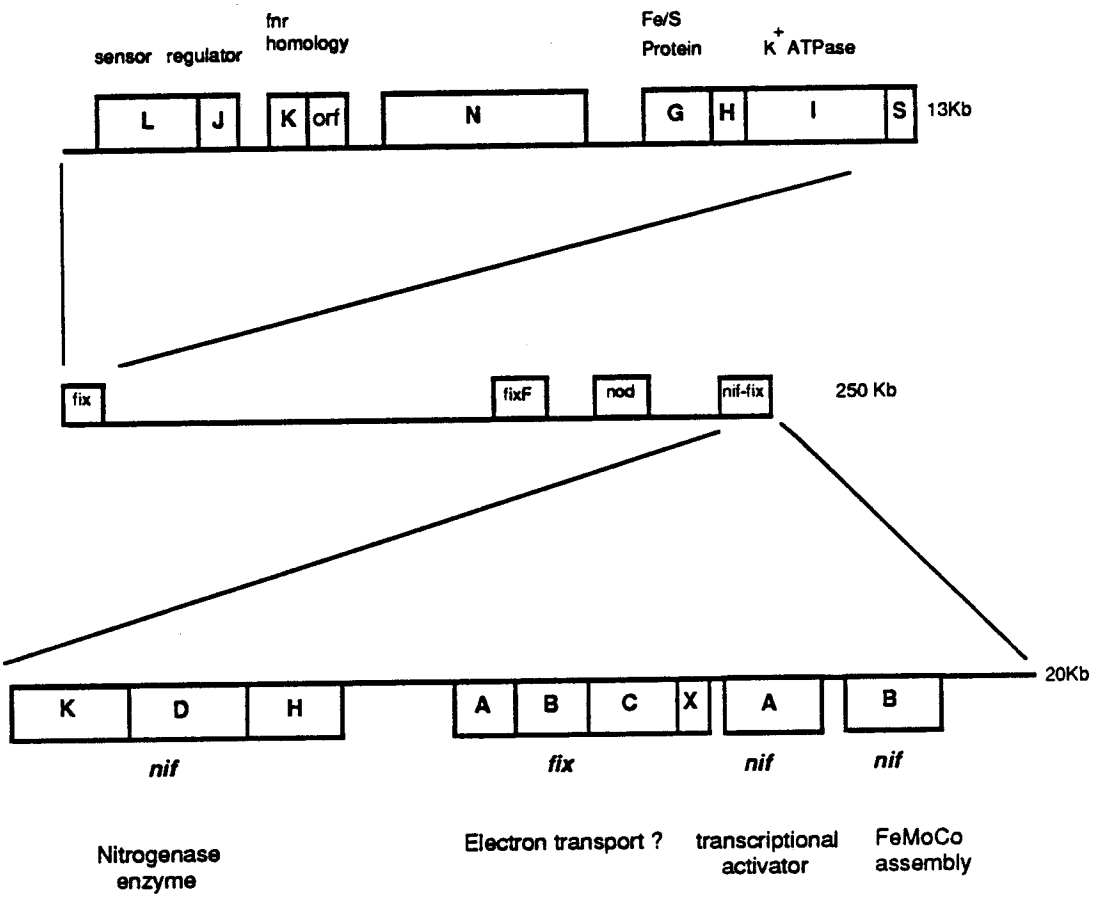
Organization of *nif* and *fix* genes in *Rhizobium meliloti.*

In contrast to *Klebsiella pneumoniae*, in which all of the nitrogen fixation genes are tightly clustered in ~25Kb of DNA (Arnold *et al.* 1988), the nitrogen fixation genes of *R. meliloti* are widely separated. Two major clusters of *nif* and *fix* genes on the *R. meliloti* pSym plasmid (Banfalvi *et al.* 1981; Charles and Finan 1990) are shown in Figure 1.

Figure 1

Nif and Fix genes of *Rhizobium meliloti*. A 250Kb section of the pSym plasmid is shown at the center. Expanded views of two regions are shown above and below. Boxes are open-reading frames for proteins. The known or proposed function of each protein is labeled (proposed functions are generally based on homology to known proteins). Nod = nodulation genes (not discussed); FeMoCo = iron-molybedenum-cofactor. The drawing scale of the expanded views above and below is approximate.

Figure 1


fnr
homology
sensor regulator
Fe/S
Protein
K+ ATPase
L
J
K
orf
N
G
H
I
S
13Kb
fix
fixF
nod
nif-fix
250 Kb
20Kb
K
D
H
A
B
C
X
A
B
nif
fix
nif
nif
Nitrogenase
enzyme
Electron transport ?
transcriptional
activator
FeMoCo
assembly

Shown at the center of Figure 1 is a 250Kb section of pSym DNA that contains two clusters of *nif-fix* genes (an expanded view of each is shown above and below). In the region between the two major *nif-fix* clusters is the *fixF (nifN)* gene. This gene, originally denoted *fixF,* was sequenced and found to encode a protein homologous to the *nifN* protein of *K. pneumoniae* (Aguilar *et al.* 1987). The genes encoding NifN (and NifB; see below), are activated by NifA and both proteins are thought to be required for the assembly of the iron-molybdenum cofactor of nitrogenase.

Shown at the bottom of Figure 1 is the *nif-fix* cluster containing the subunits of the nitrogenase enzyme (*nifHDK*). Transcribed divergently from *nifHDK* is the *fixABCX* operon (Earl *et al.* 1987). Earl *et al.* concluded that the *fixABCX* genes are involved with electron transport to the nitrogenase enzyme, based primarily on the homology between *fixX* and other bacterial ferredoxins (see also Dusha *et al.* 1987). Earl *et al.* also suggested that the amino-terminal of the FixC polypeptide contains amino acid sequences similar to leader sequences for protein export. However, I have found that the amino-terminal of FixC contains a region that is highly homologous to flavin-dinucleotide or nicotinamide-dinucleotide binding sites in many other proteins, as does a similar series of amino acids in the FixB polypeptide (data not shown). The occurence of two proteins containing nucleotide binding sites in an operon also containing a ferredoxin-like protein supports the contention of Earl *et al.*, i.e. - the *fixABCX* operon is involved with electron transport. The source of the elctrons for the nitrogenase enzyme is known for *Klebsiella pneumoniae* (FixJ, a pyruvate:flavodoxin oxidoreductase; see Arnold *et al.* 1988), but the source of electrons for Rhizobia nitro-

genase enzymes has resisted discovery. If the FixB and FixC proteins are proven to contain the nucleotide binding site, then an attractive hypothesis is FixB and FixC (and FixA?) constitue a dehydrogenase enzyme that passes electrons to *fix*X, which in turn donates the electrons to nitrogenase.

Immediately to the right of the *fixABCX* operon is the *nifA* gene, encoding the transcriptional activator (NifA) of the *nifHDK* and *fixABCX* operons (Szeto *et al.* 1984), followed by the *nifB* gene (Buikema *et al.* 1987), a protein thought to be involved with the formation of the iron-molybdenum-sulfur cofactor of the *nifDK* polypeptides of nitrogenase. Transposon mutagenesis of *fixABCX*, *nifA*, or *nifB* yields a non-nitrogen fixing phenotype (references cited above, and Corbin *et al.* 1983).

At the top of Figure 1 is a second cluster of *fix* genes. The *fixLJ* operon encodes two proteins required for the transcriptional activation of the *nifA* gene and the *fixN* gene(s) (David *et al.* 1988, and see below). *fixK*, to the right of *fixLJ*, encodes a protein homologous to the FNR protein of *E. coli* (Batut *et al.* 1989). To the right of *fix*K is the *fixGHIS* operon (Kahn *et al.* 1989), which appears by predicted protein homology to encode a cation pump required during symbioses. In this operon, the FixI protein is homologous to ATP hydrolysis driven cation exchange proteins, and the FixG protein contains a consensus amino acid sequence for an iron-sulfur cluster (Kahn *et al.* 1989).

The following sections will consider: *(i)* the structure and function of the NifA protein, *(ii)* the interaction of NifA with the promoters of nitrogen fixation genes, *(iii)* the sigma factor (NtrA) of RNA polymerase required for

nif gene expression, *(iv)* the upstream activator sequences required for *nif* gene expression, and *(v)* the regulation of *nifA* expression by FixL/FixJ.

NifA: A transcriptional regulator protein of nitrogen fixation genes.

Mutations in the *nifA* loci of *Klebsiella pneumoniae* were first systematically investigated by analysis of single-copy β-galactosidase fusions to *nif* operons (Dixon *et al.* 1980). Deletions of the *nifA* locus were found to reduce the expression of all fusions tested, with the exception of the *nifLA*::*lac* fusion. Later, the *K. pneumoniae nifA* locus was sequenced and was found to encode a protein homologous over ~60% of its length to the NtrC protein of *K. pneumoniae* (Buikema *et al.* 1985).

The *nifA* gene of *Klebsiella pneumoniae* is encoded in an operon (*nifLA)* with a second regulatory protein, NifL. NifL is homologous to the NtrB protein that regulates the activity of the NtrC (see below). In contrast to NtrB, which is both a positive and negative regulator of NtrC, only negative regulation of NifA activity by NifL has been described; deletion mutants of NifL protein do not effect the transcriptional activation activity of the NifA protein (Drummond and Wooten 1987; Arnott *et al.* 1989). The NifL protein has been found only *K. pneumoniae* ; no other nitrogen fixing bacteria appears to have NifL or a protein with NifL-like activity.

Mutations at a single locus in *Rhizobium meliloti* was also found to completely inhibit the expression of nitrogen fixation genes, as measured by the accumulation of *nif* mRNA from the "P1" and "P2" nitrogen fixation promoters (Szeto *et al.* 1984). ["P1" and "P2" are now known to be the promoters for the *nifHDK* and *fixABCX* operons]. The *R. meliloti* locus

encoding the positive control function was sequenced and found to encode a protein highly homologous to the NifA protein of *K. pneumoniae* (Weber *et al* 1985). The inital sequence of NifA (Buikema *et al.* 1985) from *K. pneumoniae* was found to be incorrect, and a second group demonstraed that over 40 codons at the carboxy-terminal of the protein were missing in the initial sequence (Drummond *et al.* 1986). The addtion of the 40 codons described in the new sequence increased the homology between *K. pneumoniae* and *R. meliloti* NifA (Drummond *et al.* 1986).

A positive regulatory locus for *nif* genes has now been identified in numerous nitrogen fixing bacteria, and the locus sequenced. In all cases, the deduced amino acid sequences are highly homologous with the NifA protein of *R. meliloti* and *K. pneumoniae.* The sequence for the NifA protein is now known for *Rhizobium leguminasarum* (Gronger *et al.* 1987), *Azorhizobium caulinodans* (Nees *et al.* 1988), *Bradyrhizobium japonicum* (Thony *et al.* 1987), *Rhodobacter capsulatus* (Masepohl *et al.* 1988), and *Azotobacter vinelandii*, which has at least three separate NifA proteins (Bennett *et al.* 1987; Joerger *et al.* 1989). The *Azotobacter vinelandii* and *A. chroococcum* (Kennedy and Robson 1983), and *R. meliloti* (Sundaresan *et al.* 1983b) *nif* genes can be activated by the *K. pneumoniae* NifA protein, demonstrating that the homology between the NifA proteins of different organisms extends to both structure and function.

The structure of NifA from different organisms has been compared and the following consensus structures can be described (references cited above and unpublished observations). The amino terminal section (~30%) of all NifA proteins are not homologous, but the carboxy-terminal 60% of all

NifA proteins are highly homologous. The carboxy region is also homologous to a number of other proteins, particulary NtrC (see below). The strongly homologous region contains a consensus ATP binding domain (Nees *et al.* 1988 and see below for the function of the ATP-binding domain in NtrC) and four conserved cysteines (in *R. meliloti, R. leguminasarum, R. capsulatus, A. caulinodans,* and *B. japonicum*; but not *K. pneumoniae* or *A. vinelandii*). The conserved cysteines may be important to all NifA proteins in which they occur; site-specific mutation of any of the cysteines destroys the transcriptional stimulation activity of the *B. japonicum* NifA (Fischer *et al.* 1988). The carboxy terminal of the NifA protein is thought to encode a DNA-binding domain, and is homologous among all NifA proteins (see Nees *et al.* 1988 for a compilation of the carboxy-terminal homology and the other regions of homology described above). However, it is not clear whether the carboxy terminal is required for activation of *nif* promoters (see Results, and Discussion).

Regulation of NifA expression.

The *nifA* gene in *Rhizobium meliloti* is regulated in two ways. First, NifA stimulation of the the *fixABCX* operon upstream of the *nifA* gene results in read-through transcription of the *nifA* gene (Kim *et al.* 1986). This read-through transcription leads to indirect auto-activation of the *nifA* gene. Second, transcription of the *nifA* gene is specifically stimulated by the FixJ protein (David *et al* 1988). The positive activation of the *nifA* promoter has been demonstrated in *Escherichia coli*, although the activation was insensitive to oxygen (Hertig *et al.* 1989). However, the oxygen insensitivety may be an artifact of the analysis conditions (see Results). The FixJ stimulated

transcription of the *nifA* gene is thought to be regulated by oxygen (Ditta *et al.* 1987; Virts *et al.* 1988; David *et al.* 1988; Batut *et al.* 1989). In addition to the positive activation of the *nifA* promoter, a second loci in *R. meliloti* has recently been described that encodes negative regulator of *nifA* promoter expression (David *et al.* 1987; Batut *et al.* 1989).

The FixLJ operon encodes two proteins that are homologous to a large family of proteins that regulate gene expression in response to environmental stimuli. In the past four years, this family of proteins has grown from the original set of 6 homologous pairs (Nixon *et al.* 1986; Ronson *et al.* 1987), to over 20 (recently and exhaustively reviewed in Stock *et al.* 1989). Stock *et al.* suggest that 50 or more sensor/regulator pairs may occur in *E. coli* simultaneously.

The family of interelated proteins consists of a paired sets of an environmental sensor protein and a transcriptional activator protein. The transcriptional activator protein is modified by the sensor and then activates (or represses) transcription from either a single operon or mutilple operons. The transcriptional regulator protein is activated by phosphorylation of a conserved aspartic acid residue, by phosphate transfer from the sensor protein (Stock et al. 1989; see also the original description of the phenomena: Ninfa and Magasanik 1986). The phosphorylated aspartic acid in the regulator, and the proposed ATP-binding site and sites of autophosphorylation in the sensor proteins are strictly conserved; it is reasonable to speculate that all sensor/regulator pairs of proteins transmit information about the external enviroment of the cell by the same phosphorylation mechanism.

The interaction of the protein pairs is baroque (Stock *et al.* 1989). For instance, one of the regulator proteins (CheY) does not activate transcription, but activates another regulator protein; the ArcA protein is probably only a negative regulator of transcription; the NarL protein is both a negative and positive regulator of transcription; the NtrB and CheA sensor proteins monitor other cytoplasmic sensors, as opposed to direct monitoring of the periplasmic environment; and some sensors of one system and the regulator of a second system can transmit information to each other by a mechanism described as "cross-talk" (Ninfa *et al.* 1988).

As noted above, the *fixLJ* operon of *R. meliloti* encodes one of these sensor/regulator pairs, where FixL is the sensor and FixJ is the regulator. The specific signal recognized by the FixL protein is unknown; it is known that FixL responds to microaerobic or anaerobic conditions (David *et al.* 1988; and see Results). When critically examined, there is also no data in the published literature that specifically identifies how any of the other external stimuli are "sensed", in addtion to the ambiguity concerning the signal for FixL. That nitrate is sensed by NarX, or osmolarity is sensed by EnvZ, or acetosyringone is sensed by VirA is well established (three of the described sensors; reviewed in Stock *et al.* 1989); the mechanism by which the external environment is monitored has so far eluded description.

The amino-terminal third of the regulator proteins (including FixJ) contains the conserved amino acid sequence that is the proposed site of phosphorylation by the sensor protein; NifA is not homologous to the regulator proteins in this region. However, NifA is highly homologous

throughout the carboxy-terminal two-thirds of its coding sequence with four proteins that are transcriptional regulators, and members of the paired sensor/regulator family of proteins: (1) NtrC of *Rhizobium meliloti* (Szeto *et al.* 1987), *Rhodobacter capsulatus* (Jones and Haselkorn 1989), *Klebsiella pneumoniae* (Drummond *et al.* 1986) and *Bradyrhizobium parasponiae* (Nixon et al. 1986), (2) DctD of *Rhizobium meliloti* (Jiang *et al.* 1989) and *R. leguminasarum* (Ronson *et al.* 1987), (3) XylR of *Psuedomonas putida* (Spooner *et al.* 1986), and (4) HydG of *Escherichia coli* (Stoker *et al.* 1989). DctD regulates the uptake of the dicarboxycylic acids succinate, fumarate, and malate; XylR regulates the catabolic pathway for xylene, and HydG regulates the anaerobic expression of formate-hydrogen lyase. In addition, NifA is homologous to the TyrR protein of *E. coli*; TyrR is a transcriptional repressor for aromatic amino acid synthesis and controls at least eight unlinked operons (Cornish *et al.* 1986). TyrR is only homologous with the carboxy terminal two thirds of NifA, and does not contain the conserved amino-terminal shared with NtrC homologs.

Within the carboxy-terminal region of homology shared between NifA,NtrC, XylR, DctD, and HydG is a consensus ATP-binding domain. The binding of ATP to the NtrC protein has been shown to be required for the isomerization of closed complexes to stable open complexes of RNA polymerase and the promoter sequence (Popham *et al.* 1989). Whether the ATP is hydrolysed during the isomerization process is not clear, but non-hydrolysable derivatives of ATP did not allow formation of stable complexes (Kutsu *et al.* 1989). It is not known if the proposed ATP binding site in NifA (and DctD, XylR, or HydG) functions in a similar manner.

Surprisingly, there is an even deeper level of relationship between NtrC, DctD, XylR, HydG, and NifA. In addition to amino acid sequence homology, each of the five proteins regulates genes or operons whose promoters are recognized by the same unique sigma factor for RNA polymerase, NtrA (reviewed in Kutsu *et al.* 1989).

NtrA: A special sigma factor for transcription of *nif* genes.

Mutations in the *ntrA* locus of *Salmonella typhimurium* and *Escherichia coli* exhibit a strict auxotrophic requirement for glutamine (reviewed in Reitzer and Magasanik 1987). Subsequently, the NtrA protein was proven to be a sigma factor for RNA polymerase (Hirschman *et al.* 1985). NtrA-associated RNA polymerase recognizes a different sequence of nucleotides as a transcriptional promoter, than does the most abundant sigma factor of Enterobacteria, sigma^{70} (Hirschman *et al.* 1985). The sequence of NtrA promoters in enterobacteria is highly homologous to the promoters of nitrogen fixation genes in *Klebsiella pneumoniae* and *Rhizobium meliloti* (references cited in Reitzer and Magasanik 1987; and see Kutsu *et al.* 1989). The initial mapping of the start sites of transcription for the *K. pneumoniae* and *R. meliloti nifHDK* genes showed the two promoters to be highly homologous, but the relationship to NtrA stimulated promoters was not noted (Sundaresan *et al.* 1983).

Since the original description of NtrA as a sigma factor for enterobacteria (only four years ago), NtrA has been shown to control a multitude of others genes and the *nif* genes of most (but not all) prokaryotes (Thony and Hennecke 1989). Of the nitrogen fixing bacteria, NtrA has been cloned

and sequenced in *Azotobacter vinelandii* (Merrick *et al.* 1987), *Klebsiella pneumoniae* (Merick and Gibbons 1985), and *Rhizobium meliloti* (Ronson *et al.* 1987). In each of these bacteria, the NtrA protein has been shown to be essential for both nitrogen assimilation and nitrogen fixation, and nitrate assimilation in *R. meliloti* (references cited above).

NtrA has been shown to be the essential RNA polymerase sigma factor for transcription of a constantly increasing number of promoters. In addition to the *dct, xyl, hyd, nif*, and *ntr* operons (of various species) discussed above, NtrA controls diverse metabolic pathways in *Alcaligenes eutrophus* and *Psuedomonas facilis* (Romerman *et al.* 1989), flagellar genes in *Caulobacter crescentus* (Ninfa *et al.* 1989; Mullin and Newton 1989), various metabolic pathways in *Psuedomonas putida* (Kohler *et al.* 1989), and anaerobic metabolism in *Escherichia coli* (Birkmann *et al.* 1987). None of these bacteria fix nitrogen.

Upstream activator sequences are required for *nif* gene expression.

The final topic to be reviewed is the role of "upstream activator sequences" for the activation of nitrogen fixation genes. Buck et al. were the first to describe a region of DNA homology upstream of nitrogen fixation promoters (Buck *et al.* 1986). They described a characteristic sequence of nucleotides that occurs approximately 100 bp upstream of the *nifH* promoter in *Rhizobium trifolii, R. meliloti, R. phaseoli, Bradyrhizobium japonicum, Parasponium rhizobium, Azotobacter chroococcum, A. vinelandii*, and the *nif H, U, B,* and *E* promoters of *Klebsiella pneumoniae.* The essential results of their work are: *(i)* the conserved sequence does not serve as a

promoter, and *(ii)* the orientation of the sequence and distance upstream from the promoter is not critical (see also Buck *et al.* 1987). This sequence has continued to appear whenever a NifA activated promoter is mapped, although it is not always noted by the authors (personal observation). The activator sequence has been shown by *in vivo* methylation analysis to be the binding site of NifA (Morett and Buck 1988) and the site may be involved with the formation of loops of DNA between the NifA protein and the NtrA-RNA polymerase, at least in *K. pneumoniae* (Buck *et al.* 1987). With regards the experiments described in this work, the *nifB* promoter of *R. meliloti* contains a consensus upstream activator sequence (Buikema *et al.* 1987). Buikema *et al.*suggests that there may be two activator sequences upstream of *nifB*, but the promoter proximal sequence does not match the consensus of other *R. meliloti* sequences (personal observation; data not shown).

Escherichia coli anaerobic metabolism.

The analyses of the *Rhizobium meliloti nifA* and *nifB* promoters described below was conducted by gene fusion analysis in *Escherichia coli.* Therefore, it is appropriate to include a short review of *E. coli* anaerobic metabolism.

The anaerobic metabolism of *E. coli* is well studied, particulary nitrate respiration and anaerobic fermentation (see Clark 1989; Lin 1987; and Stewart 1988 for a comprehensive reviews). However, a complete description of all anaerobically regulated genes is far from complete and the transcriptional regulation of anaerobically expressed operons is much less

advanced (although more is known about facultative anaerobic metabolism of *E. coli* than any other organism)

Clark has presented gene fusion evidence for at least 50 anaerobically regulated genes in *E. coli* (Clark 1984) and a similar estimate has been made by analysis of individual proteins (Neidhardt 1983). Some of the anaerobically regulated genes are difficult to reconcile with a presumed increased requirement during anaerobic growth; leucine, valine/isoleucine, thiamine, and tyrosine operons have all been found to have as much as 10-fold higher expression anaerobically (Reams *et al.* 1987), as has the *aidB* gene induced in response to DNA methylating agents (Volkert *et al.* 1989). Why any of these operons would require higher expression during anaerobic growth is obscure. Recently, a novel anaerobic ribonucleotide-triphosphate-reductase has been discovered in *E. coli* (Fontecave *et al.* 1989), demonstrating that even an area of research as well studied as the nucleotide metabolism of *E. coli* still holds suprises when anaerobic metabolism is investigated.

The genetics of anaerobic gene regulation in *E. coli* is complex. Numerous transcriptional regulators control the expression of anaerobic metabolism, but two primary sources of control are known. The *arcA* and *arcB* proteins, members of the two-component family of environmental sensors and gene regulators, regulate the shift from aerobic metabolism to anaerobic metabolism (Iuchi and Lin 1988; Iuchi *et al.* 1989a, Iuchi *et al.* 1989b). The ArcA protein, the regulator of the two-component system, is a transcriptional repressor of aerobically expressed operons; to date, no transcriptional activation activity has been described for ArcA. The second

important transciptional regulator of anaerobic gene expression is the FNR protein (reviewed in: Stewart *et al.* 1988; Lin 1987), required for expression of anaerobic respiration operons. FNR also controls an anaerobic asparaginase enzyme (Jennings and Beacham 1990); the relationship between asparaginase and anaerobic respiration is obscure.

The transcriptional control of fermentative operons is far less well described than control of anaerobic respiration, but numerous regulatory loci have been described, although not yet thoroughly investigated. (see Aliabadi *et al.* 1988). No *Escherichia coli* anaerobic transcriptional regulator is known to be essential for heterologous *nif* gene expression in *E. coli*. However, *E. coli* NtrC can stimulate *nif* fusions when ammonia is limiting (Sundaresan *et al.* 1983).

The experimental design of the work described in the following sections is two-fold. First, assemble a sensitive system for the analysis of the *nifA* and *nifB* promoters. This system will depend on the construction of β-galactosidase gene fusions to the two promoters and placement of a single copy of the fusions in the *E. coli* chromosome. Second, determine the activity of the two promoters under rigorously defined physiological conditions. The essential physiological analysis will be to compare the activity of *nif* promoter expression during aerobic and anaerobic respiration, and during anaerobic fermentation with different carbon sources.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The genotypes *E. coli* K-12 strains, bacteriophages, and plasmids used in this study are shown in Table 1. Stock cultures were maintained in LB + 25% glycerol at -60 °C. Frozen stock cultures were streaked to solid media without thawing and returned immediately to storage. Bacteriophage lambda (λ) stocks were stored as chloroform-sterilized lysates at 2 °C. Plasmids were stored as purified DNA in TE buffer (10mM Tris, 1 mM EDTA, pH 8.0) at 2 °C, and in various *E. coli* K-12 strains in frozen storage.

The genotypes of all strains were verified by testing for the selectable markers listed in the genotype. DH5α was tested for lactose auxotrophy and ampicillin sensitivity in the absence of pUC19, and nalidixic acid resistance. MC4100 derivatives (SG21155, PE55, PE56) were tested for fructose, ribose, lactose, and arabinose auxotrophy, streptomycin resistance, and λ phage, ampicillin, and kanamycin sensitivity. P90C was verified to contain lactose auxotrophy. JC10289 was tested on minimal sorbitol plates for sorbitol auxotrophy and on LB plates for UV sensitivity. Strains constructed for this work were tested for the introduced mutation (see Strain construction).

Throughout this manuscript, italicized three-letter genotype mnemonics refer to specific genes (*i.e.-nifA* is a genetic locus) and capitalized mnemonics designate proteins (*i.e.*-NifA is the predicted protein of the sequenced *nifA* gene). This format names proteins by their genotype

mnemonics, when the amino acid sequence and characteristics of a protein is known solely from DNA sequencing.

All strains were purified by streaking for single colonies at least twice before storage or genetic manipulation. Standard methods of bacteriology were used to determine cell number, culture optical density, phage titers, and for propagation of plasmids and phages (Miller 1972; Silhavy *et al.* 1984).

Strain construction

Initial attempts to overproduce the NifA protein in *E. coli* K-12 strains that contained a wild-type Lon protease suggested NifA is sensitive to the Lon protease (data not shown). Therefore, a *lon lac* strain of *E. coli* (SG21155; seeTable 1) was obtained for physiological experiments in which NifA was used to stimulate β-galactosidase activity from gene fusions. This strain contains a genetically engineered deletion in the Lon protease gene (Maurizi *et al.* 1985). Commonly used *lon* strains of *E. coli* K-12 contain the *lon*100 mutation, often incorrectly identified as Δ*lon*100. This is not a deletion of the Lon gene, but probably an insertion in the *lon* promoter region (Maurizi *et al.* 1985). The mutation is unstable and often reverts to wild type. Two popular *E. coli lon lac* strains, Y1089 and Y1090 (used with λgt phages) are reported to have the "Δ*lon*100" mutation. However, none of the pleiotropic effects of *lon* mutations were detected in these strains (data not shown) and they were not used .

Table 1. Bacterial strains, phages, and plasmids

E. coli strain	Genotype	Source
P90C	*ara*, Δ(*lac-pro*), *thi*	R. Simons
DH5α	F-, ϕ80d*lacZ*ΔM15, *endA*1, *recA*1, *thi*1, *hsdR*17, *supE*44, *gyrA*96, *relA*1(?)	BRL, Inc.
SG21155	F-, *araD*139, Δ(*argF-lac*)U169, *relA*1, *flbB*5301, *deoC*1, *ptsF*25, *rpsL*150, *relA*1, Δ*gal*, Δ*lon*	S. Gottesman
PE55	as SG2115 and *sulA*	this work
PE56	as PE55 and *recA*	this work
PE5645	as PE56 and λRS45	this work
AZ45	as PE56 and λAZ45	this work
BZ45	as PE56 and λBZ45	this work
ABZ45	as PE56 and λABZ45	this work
RAZ45	as PE56 and λRAZ45	this work

Plasmids and Phages	Source
pUC19	BRL, Inc.
pRS550	R. W. Simons, UCLA
pTTQ8	Amersham, Inc.
λRS45	R. W. Simons, UCLA
λNK330	R. W. Simons, UCLA
λNK395	R. W. Simons, UCLA

1. Construction of PE55. Mutations affecting the *lon* locus, which encodes an ATP dependent protease, are pleiotropic; cells containing reduced Lon activity are *(i)* sensitive to unknown factors in rich media, *(ii)* mucoid, *(iii)* exhibit lethal filamentation after exposure to UV light, and *(iv)* produce essentially clear plaques of lambda phage (Maurizi *et al.* 1985). The physiological experiments planned for this work required λ lysogens growing in defined rich media, two conditions senstitve to *lon* mutation. Therefore, a mutation compensatory to *lon* was selected.

Approximately 1 x 10^7 SG21135 cells were plated on LB media containing 0.05% ethylmethane sulfonate (EMS) and incubated at 30 °C. EMS selects for mutations at the *sulA* and *sulB* loci that phenotypically suppress lon mutations (Gottesman *et al.* 1981). 50 EMS resistant colonies were picked onto fresh EMS plates containing 15 g NaCl /L. Two colonies were found to be salt-sensitive and were discarded, the rest showed no salt effect (*sulB* mutations are salt-sensitive and less compensatory to *lon* than *sullA*, Gottesman *et al.* 1981). The EMS resistant cells showed reduced spontaneous filamentation when viewed by reverse-phase microscopy, were at least 10-fold more UV resistant (not quantified), and produced turbid plaques of lambda phage. One of presumed *sulA* EMS resistant colonies was purified by streaking twice for single colonies and designated PE55.

2. Construction of PE56. Lysogens of wild-type lambda phage are not entirely stable. The supernatant of overnight lysogenic culture will have approximately 1x10^6 phage/mL (Silhavy *et al.* 1984). These phage occur

from *recA*-mediated spontaneous release of lysogenic repression. The nitrogen fixation promoter fusions analysed in these experiments are integrated into the *E. coli* chromosome as recombinant, lysogenic phage. In addition, the lysogenized cells contain various plasmids with DNA homologous to the integrated phage. Therefore, two problems can arise in recombination proficient strains - (i) recombination between the multi-copy plasmids and the single-copy phage that would generate unregulated fusions, and (ii) a sub-population of cells with mutiple copies of the fusion generated by spontaneous phage induction. Therefore, creation of a *recA* derivative of PE55 would eliminate both recombination between the plasmids and lysogens and spontaneous release of the lysogen (Csonka and Clark 1979).

To generate a *recA* derivative of PE55, a culture of PE55 was transduced with a phage P1 lysate (Silhavy *et al.* 1984) grown on *E. coli* strain JC10289 (*recA*::Tn10 [tet^r]) (Csonka and Clark 1979). All tetracycline resistant colonies produced were exceedingly UV sensitive (at least 100 fold more sensitve than PE55, data not shown). Twenty colonies were picked to a minimal sorbitol plate and all were found to be sorbitol auxotrophs, as expected (Csonka and Clark 1979). One colony was selected, purified, and designated PE56.

Cultures of PE56 containing plasmids encoding ampicillin resistance repeatedly lysed in liquid culture; however, the effect was variable and occurred arbitrarily from week to week. The problem was finally traced to ampicillin.

Two separate ampicillin effects were discovered. Cultures of *lon* strains PE56, PE55, and SG21155 are killed in liquid culture by 0.05 μg/mL ampicillin; in contrast, the Lon$^+$ parent of SG21155, MC4100, can survive a 20-fold higher ampicillin concentration (data not shown), demonstrating that at least these *lon* strains are hyper-sensitive to ampicillin. In addition, plates with as little as 1μg/mL of ampicillin became toxic to PE56 cultures containing plasmid encoded ampicillin resistance, if stored for more than a few days at room temperature, or more than a week at 4 °C. When stored plates were streaked with ampicillin^r PE56, numerous ampicillin^s papillae arose. Presumably, the lysis of liquid cultures was due to inoculation with colonies containing ampicillin^s papillae (the papillae are only visible under magnification, and so were not apparent). Control experiments showed that PE56 cultures containing an amp^r plasmid will inherit the plasmid stably in the absence of ampicillin selection (data not shown); therefore, ampicillin was omitted from liquid culture physiological experiments.

No literature reference could be located which specifically addressed the question: Do anaerobicly grown cultures *recA* cells maintain lysogens of λ phage in a stable state, as do aerobic cultures (see above)? Or, in a more general sense, does *E. coli* have an anaerobically regulated recombination system in addition to *recA* that will stimulate the release of lysogenic phage? Although the λ vectors used in these experiments have also been used to investigate the anaerobic physiology of *E. coli* (see Discussion), none of the published papers mentioned testing *recA* cells for <u>anaerobic</u> stability of the lysogen.

No spontaneous release of phage from PE56 during either anaerobic fermentation in glucose or xylose media, or anaerobic respiration in glucose-nitrate or xylose-nitrate media was found. As a control, aerobic *recA* cultures in the same media produced $<1x10^9$ phage/mL, as reported (Silhavy *et al.* 1984; Simons *et al.* 1987). These results demonstrated that lysogens derived from λRS45 (Table 1) are stable in *recA lon* cells, both aerobically and anaerobically.

Media

Media for aerobic growth of cultures for plasmid purification was Luria broth (LB; 10g/L tryptone, 5g/L yeast extract, 5 g/L NaCl). Lambda phage were grown in LB was supplemented with 10mM $MgSO_4$ from a 1M filter sterilized stock (λLB); maltose was omitted. Competent cells for transformation were grown in SOB media (20 g/L trptone, 5g/L yeast extract, 10 mM KCl, 20mM $MgSO_4$), prepared exactly according to Hanahan (Hanahan 1983). Minimal media was M63 (Silhavy *et al.* 1984) with the appropriate carbon source and auxotrophic supplements as required.

For anaerobic growth, a nutritionally complete media was specifically developed, based primarily on MOPS media (Neidhardt *et al.* 1974). It contained, in (millimoles/liter): alanine (0.8), arginine (5.2), asparagine (0.4), aspartate (0.4), cysteine (0.1), glutamic acid (0.6), glutamine (0.6), glycine (0.8), histidine (0.2), isoleucine (0.4), leucine (0.8), lysine (0.4), methionine (0.2), phenylalanine (0.4), proline (0.4), serine (10.0), threonine (0.4), tryptophan (0.1), tyrosine (0.2), and valine (0.6); adenosine, uridine, guanosine, thymidine, and cytidine (0.2 each), and thiamine, calcium

pantothenate, p-aminobenzoic acid, nicotinic acid, and biotin (0.02 each). These reagents were prepared as a10-fold (10X) concentrate, pH adjusted to 7.2 with potassium hydroxide, filter sterilized, and stored at 2 °C.

The modified MOPS basal media contained (in millimoles/liter): potassium phosphate (2.0), magnesium sulfate (0.5), iron sulfate-heptahydrate (0.05), MOPS (100.0), Tricine (4.0), lactic acid (5.0) and micronutrients. Micronutrients is a modified mixture of essential metals (Neidhardt *et al.* 1974). It contained (in micromoles/liter): sodium molybdate, sodium selenate, boric acid, zinc sulphate, nickel chloride, manganese chloride, and calcium chloride (1.0 each), and copper sulphate (0.01). The concentration of molybdenum was increased over Neidhardt's formula to ensure an ample supply of the metal during anaerobic respiration with dimethyl sulfoxide, trimethylamine-N-oxide, or nitrate (Lin 1987). Neidhardt did not specify the addition of selenium or nickel; these were added to ensure a supply of selenium for formate dehydrogenase and pyruvate-formate lyase, and nickel for hydroenase (Lin 1987).

Complete anaerobic media (CAM) was prepard by adding MOPS (free acid form) as a solid and 100X solution stocks (pH 7.4) of lactic acid, phosphate, Tricine/iron sulphate, and micronutrients to double-distillled H_20 to make a solution of 80% final volume. A carbon source was added either as a solid sugar, or as a 100X stock solution of a non-fermentable carbon (glycerol or lactate). The final concentrations of the various carbon sources were: glucose, mannitol, maltose, or pyruvate (50 mM); glycerol, lactate, and xylose (100 mM). For anaerobic respiration media, potassium nitrate was added as a solid to a final concentration of 20mM; trimethylamine-N-

oxide (TMANO) (Sigma Chemicals) was added as a solid to 50mM; potassium fumarate was added from a 1 M stock solution, pH 7.0, to a final concentration of 100 mM. In experiments where the effect of fumarate respiration was compared to nitrate or or TMANO respiration, 200 mM KCl was added to the nitrate and TMANO media to compensate for any osmotic effects of the fumarate. 10X AVN was added last, the entire mixture adjusted to pH 7.4 with KOH, then brought to final volume with water, and filter steirilized. The media was stored at 2 °C. The supplemented MOPS media of Neidhardt, essentially the same as CAM with respect to major components, will support growth of wild-type *E. coli* with a 21 min generation time, the same as undefined rich media such as LB + glucose (Neidhardt).

During the completion of these experiments, it was found that media containing potassium nitrate became discolored (slightly yellow) if stored at 4 °C. for greater than two weeks, or left at room temperature for more than a few days. Therefore, nitrate respiration experiments were conducted with freshly prepared media. The discolored media was found to give anaerobic growth rates approximately 20-30% slower than fresh media, and the effect increased upon storage. The source of the effect is unknown.

Conditions for Aerobic and Anaerobic Growth.

Cultures for preparation of plasmids and phages were grown in an air incubator at 37 °C. with rotary shaking of at least 300 rpm. The flask volume/culture volume ratio was always at least 10. Cultures were routinely cooled on ice before centrifugation at >6000x g. for 10 minutes at 0 °C.

For experiments where the aerobic value of a *nif::lac* gene fusion was measured, the cultures were grown at 30 °C. in a temperature regulated water bath, with a rotary shaking speed of >300 rpm. Baffled culture flasks with Morton closures were used with a culture volume of less than 5% of the flask volume. Overnight cultures were diluted 1:1000 into CAM media with the desired carbon source and grown until the optical density (OD_{550}) was ~0.1, then diluted 1:100 into identical pre-warmed media and the growth continued until the OD_{550} was ~0.2. To collect the samples, a 100 mg/mL solution of chloramphenicol in absolute ethanol was added to a final concentration of 100 µg/ml as the flask was removed from the shaker and rapidly rotated by hand in an ice/water bath for at least 5 minutes. The culture was then centrifuged at 6000x g for 10 minutes at 0 °C. These cells were placed on ice as a 20-fold concentrate of the original culture volume in Z buffer (see Enzyme Assay for β-Galactoside, below) until used.

For anaerobic growth of cells, overnight cultures were grown aerobically (in various media, see Results) at 30 °C. in an air incubator, with rotary shaking >300 rpm. Transfer to anaerobic conditions was done as required by the conditions of the experiment, and will be discussed below (Results). The media for anaerobic growth experiments was exclusively CAM, with the addition of a desired carbon source, anaerobic respiration source, or chemical addition necessary for the separate experiments.

Anaerobic cultures were grown at 30 °C. in 15 mL test tubes, overlayered with light paraffin oil, in a temperature regulated water bath. The water in the bath was constantly recirculated and maintained at the assay

temperature to within 0.2 °C. The recirculating pump for the bath directed water through a small turbine-driven, submersible magnetic stirrer. The cultures tubes each contained a sterile micro-stir bar (Teflon covered permanent magnet approximately 1 x 5 mm). The culture tubes were held in place on the stirring turbine by a plastic rack. This apparatus gave continuously stirred anaerobic cultures maintained at a constant temperature. The stirring was not strong enough to visibly disturb the oil-water interface.

Anaerobic conditions were maintained by the addition of 0.1 units of Oxyrase (Oxyrase Inc., Ashland, OH) per mL of culture. Oxyrase is a cytoplasmic membrane preparation of *E. coli* cells, containing an intact oxygen respiration system. The addition of millimolar concentrations of either succinate or lactate to media containing Oxyrase supplies reducing equivalents (through succinate dehydrogenase or lactate dehydrogenase) to the cytochrome oxidase proteins and the subsequent reduction of oxygen to water. At 0.1 unit-/ml, Oxyrase will reduce the dissolved oxygen concentration in solution to zero in 2-4 minutes (manufacturers specifications). More importantly for this work, the reagent allows repeated sampling of an anaerobic culture without the use of an anaerobic growth chamber or elaborate inert gassing systems.

The activity of Oxyrase Lot #91118 was tested according to the manufacturers instructions and found to be fully active (data not shown). [The analysis was done in the laboratory of Dr. Norman Bishop, OSU Dept. Botany, to whom the author is indebted for his gracious assistance]. Other lots of Oxyrase were tested; not all were active. The initial lot of reagent, although still within the reported shelf-life, was found to be less the 5% active.

Three lots of Oxyrase were contaminated with an *E. coli* specific phage (titer ~1000/mL) capable of replication and infection of anaerobic and aerobic cultures. All experiments were done with phage-free Lot #91118, supplied free-of-charge by the manufacturer, after we alerted them to the presence of the phage. The phage will infect F^+ and F^- *E. coli*, lysogens of λ phage, and *recA* strains. Divalent cations are not required for infection by the phage, the plaques are large and clear and appear within three hours of plating on*E. coli* lawns, and aerobic titers of the phage reach 1×10^{11} (data not shown). These analysis are consistent with the characteristics of small icosohedral coliphages such as MS2 or ϕX174. This information is presented only to alert other investigators to the presence of the phage; unfortunately, the supplier has found the phage to contaminate most of their lots of reagent (personal communication). Until the problem is solved, the use of this (in other ways excellent) reagent, must be accompanied by due caution.

One final note of caution. Oxyrase has been used for the isolation and growth of many facultative and strict anaerobic bacteria (literature references supplied on request by the manufacturer). On two separate occasions, anaerobic cultures of BZ45/pNF19 were contaminated with an anaerobic bacteria. The culture produced extensive gas from glucose or maltose; visual inspection of the culture by reverse-phase microscopy showed numerous large cocci. Extensive testing of all reagents and equipment finally localized the source of contamination to one set of frozen stock cultures. The contamination was proved by further testing to have derived from a single bottle Luria broth-25% glycerol storage media. The

media was not visibly contaminated and did not produce colonies when plated aerobically. A reasonable explanation for this distressing series of events is that heat-resistant spores of an anerobic bacteria survived the autoclaving procedure and could be grown in complete media when Oxyrase was added. The infected cultures were discarded, fresh storage media was prepared (autoclaved then filter-sterilized), and all other stored cultures checked for contamination and re-frozen. Obviously, supposedly "pure" cultures of aerobic bacteria can be insidiously contaminated with anaerobic bacteria, and physiological experiments conducted under strict anaerobic conditions provided by Oxyrase and growing cultures of *E. coli* must be rigorously monitored for contamination.

DNA Cloning - General Methods

Standard methods of molecular biology were used for manipulation of DNA (Maniatis *et al.* 1982; Silhavy *et al.* 1984; Perbal 1988). Restriction enzymes, ligase, lambda phage DNA for molecular weight standards, and nucleases were from New England Biolabs, and were used according to the manufactors instructions. Agarose for electrophoresis was Genetic Technology Grade SeaKem agarose, and SeaPlaque low gelling temperature agarose, both from FMC. DNA restiction fragments to be cloned were purified by electrophoresis through low gelling temperature agarose, and isolated as follows: the DNA fragment, visualized with minimal exposure to long-wavelength UV light, was excised from the gel with a razor and melted in a microcentrifuge tube at 65 ^{0}C. An equal volume of buffer saturated phenol (Maniatis *et al* 1982) was added, vortexed, and frozen at -70 ^{0}C. for 5 min-utes. The sample was then centrifuged for 3-4 minutes (until just

melted), the upper aqueous phase was removed and 0.5 volumes of saturated ammonium acetate was added and the DNA precipitated and centrifuged (Perbal 1988). This method routinely gave essentially complete recovery of the DNA, and excellent ligation to plasmid vectors.

Mini-preparatios of plasmids for restriction analysis were prepared according to Birnboim, (Birnboim 1983) with the following modifications: 5M ammonium acetate was used in place of LiCl for RNA precipitation; 0.6 volumes of isopropanol was used in place of 2 volumes of ethanol for DNA precipitation; RNAase digestion was omitted.

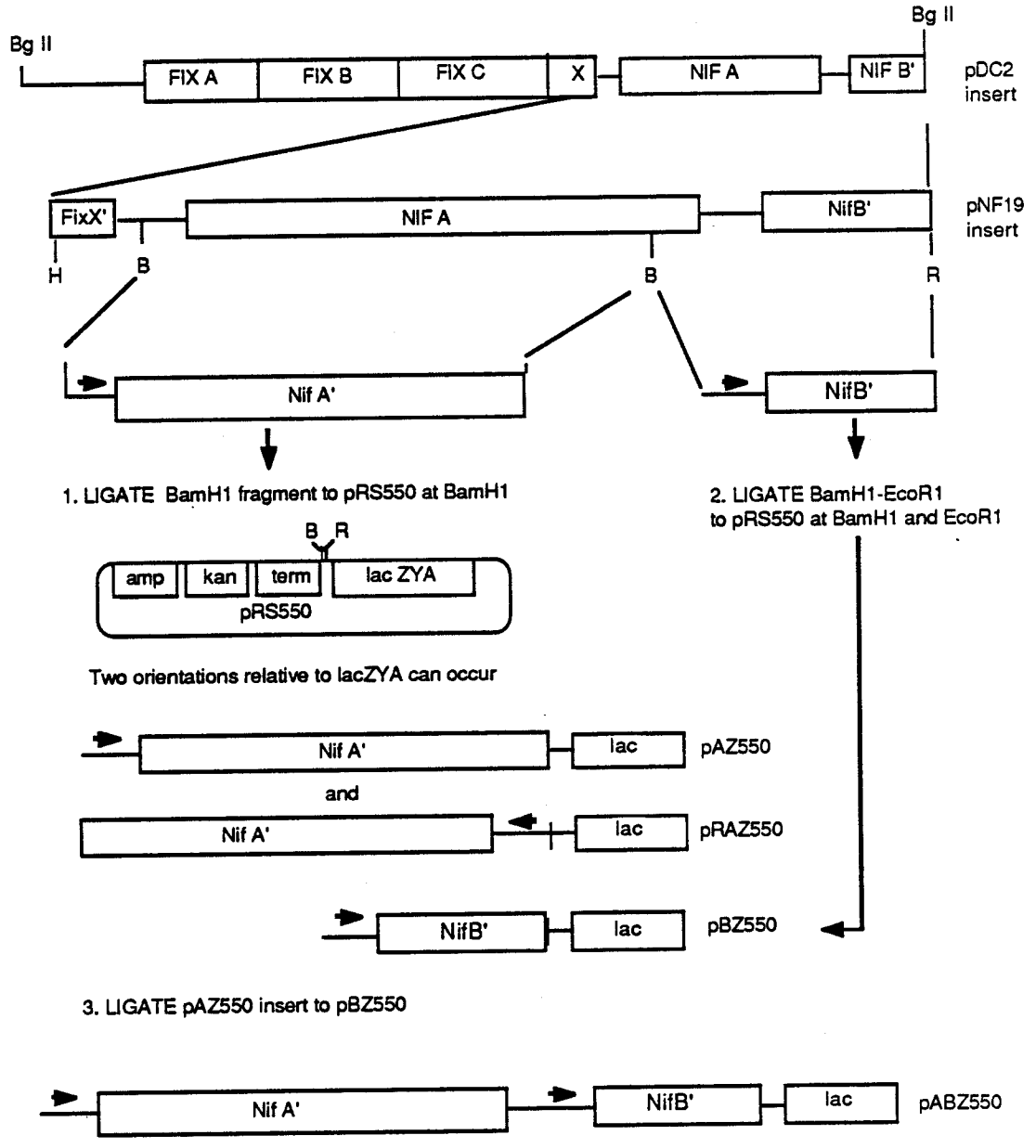
Constructed plasmids were verified by restriction analysis of plasmid mini-preparations. Each construction was verified by any or all of the following analysis, as required: *(i)* occurrence of new restriction sites unique to the insert, *(ii)* deletion of any plasmid restriction sites by ligation of a complementatry but non-identical restriction site, *(iii)* length of the inserted DNA, *(iv)* identity of the plasmid vector by restiction with an enzyme giving multiple bands diagnostic for the vector, *(v)* length of the linearized, recombinant plasmid and *(vi)* length of restriction fragments unique to the inserted DNA. Electrophoretic conditions that allowed precise measurement of fragment length were used (long gels with slow electrophoresis times); equivocal measurements were always repeated with either a new enzyme reaction and/or greater electrophoretic separation.

Competent cells were prepared according to Hanahan (Hanahan 1983), omitting the DTT addition. Lambda phage was manipulated using standard techniques (Silhavy *et al.* 1984; Simons *et al.* 1987).

FIGURE 2

Construction of pNF19 and pRS550 lac fusion plasmids. The major cloning steps for the construction of pNF19, pBZ550, pAZ550, pRAZ550, and pABZ550 is shown. pNF19 was formed by ligation of the *Hin*D III-*Bgl* II fragment of pDC2 to pUC19 at *Hin* D III and *Bam* HI. The *Eco* RI site in pUC19 next to the *Bam* HI/*Bgl* II junction was then used for subsequent cloning steps. The operon fusions pBZ550, pAZ550, pRAZ550, and pABZ550 were constructed by ligation of pNF19 restriction fragments to pRS550, as shown. Amp, ampicillin resistance gene; kan, kanamycin resistance gene; term, re-iterated transcriptional terminators of pRS550 (Simons *et al.* 1987); lac and lac ZYA are the *E. coli* β-galactosidase operon; X, FixX. FixX' and NifB' are truncated segments of the *fixX* and *nifB* genes, predicted from published sequences. Boxed regions are the open reading frames of the various genes. The arrows preceding the *nifA* and *nifB* genes show the promoter location and direction of transcription. Restriction sites are: Bg, *Bgl* II; H, *Hin* DIII; R, *Eco* RI.

Figure 2


Bg II
Bg II
FIX A
FIX B
FIX C
X
NIF A
NIF B'
pDC2
insert
FixX'
NIF A
NifB'
pNF19
insert
H
B
B
R
Nif A'
NifB'
1. LIGATE BamH1 fragment to pRS550 at BamH1
2. LIGATE BamH1-EcoR1
to pRS550 at BamH1 and EcoR1
B R
amp
kan
term
lac ZYA
pRS550
Two orientations relative to lacZYA can occur
Nif A'
lac
pAZ550
and
Nif A'
lac
pRAZ550
NifB'
lac
pBZ550
3. LIGATE pAZ550 insert to pBZ550
Nif A'
NifB'
lac
pABZ550

DNA Cloning: Plasmid Construction

A. Sub-cloning of the *nifA-nifB* region. Plasmid pNF19 was constructed by isolating the 2.9 Kb. *Hin* DIII - *Bgl* II band of pDC2 (Corbin *et al.* 1983) and ligating it to pUC19 (Table 1) at the *Hin* DIII and *Bam* H1 sites (Figure 2). Repeated restriction analysis failed to detect the *Eco* R1 restriction site shown in either of two published *nifA* sequences (Benyon *et al.* 1988; Szeto *et al.* 1984). The orignal description of pDC2 does not show the *Eco* R1 site common to other isolates of *R. meliloti* either (Corbin *et al.* 1983). This construct (and all other plasmid constructions) was transformed to *E. coli* DH5α for initial isolation and restriction analysis of recombinants. After verification of the construction, the plasmid was re-purifed and stored for transfer to other strains.

B. Construction of operon fusions to β-galactosidase. Plasmid pBZ550 was constructed by isolating the 946 bp *Bam* H1-*Eco* R1 band of pNF19 and ligating it to pRS550 (Simons *et al.* 1987) at the *Bam* H1-*Eco* R1 sites (Figure 2). The *Eco* R1 restriction site of pNF19 is derived from the polylinker region of the plasmid; the *Bam* HI/*Bgl* II junction of pNF19 10 bp from the *Eco*R1 site cannot be recut by *Bam* H1 or *Bgl* II and is not shown.

Plasmid pAZ550 was constructed by isolating the 1621 bp *Bam* H1 fragment of pNF19 and ligating it to pRS550 at *Bam* H1 (Figure 2). The orientation of the insert was verified by *Xho*1 digestion at the site in the amino-terminal region of NifA and the *Xho*1 site of pRS550. The ligation reaction for pAZ550 produced ampicillin^r, kanamycin^r colonies of two phenotypes on Xgal, light blue and darker blue. Suprisingly, the darker

blue colonies did not contain the *nifA* promoter fusion, but had the proper insert in the reverse orientation. The lighter blue colonies contained the desired insert in the proper orientation. The plasmid producing the darker blue colonies was saved as pRAZ550 ('R' designating reverse).

Plasmid pABZ550 was constructed by ligating the 1621bp *Bam* H1 fragment of pNF19 to pBZ550 at the *Bam* H1 site (Figure 2). The orientation of the insert was verified by Xho1 restriction analysis as for pAZ550.

C. Constuction of expression vector clones of NifA. To express the NifA protein in *E. coli,* the plasmid expression vector pTTQ8 was used (Stark 1987). Early attempts to express the NifA protein from a variety of other vectors were unsuccessful; no recombinant plasmids were isolated that contained the NifA protein coding region in the proper sense for expression (data not shown). Stark has shown that commercially available expression vectors that depend on a single chromosomally encoded lactose repressor for control of a strong *E. coli* promoter are, effectively, unregulated (Stark 1987). By constructing the TTQ series of vectors, that contain the LacI repressor gene, Stark was able to create plasmids that are tightly regulated and capable of accepting DNA that codes for proteins lethal to *E. coli.* The TTQ8 plasmid was therefore chosen to attempt regulated cloning of the NifA protein. These experiments were successful.

During initial work with the TTQ8 plasmid, no α-complementation between the α-peptide encoded in pTTQ8 and the chromosomal *lac* M15-deletion of DH5α was visible in colonies plated with the chromogenic indicator Xgal, although Stark describes very low, but detectable, activity

(Stark 1987). However, the flourogenic lactose indicator MUG (methyl-umbelliferone-β-D-galactoside) in LB plates (at .01mg/mL) allowed easy differen-tiation between DH5α colonies containing the TTQ8 plasmid and those without the plasmid. When viewed under long-wave-length UV light, the plasmid containing strains were brightly flourescent on MUG plates, the controls were non-flourescent. MUG plates were therefore used to verify further cloned constructs of the NifA gene.

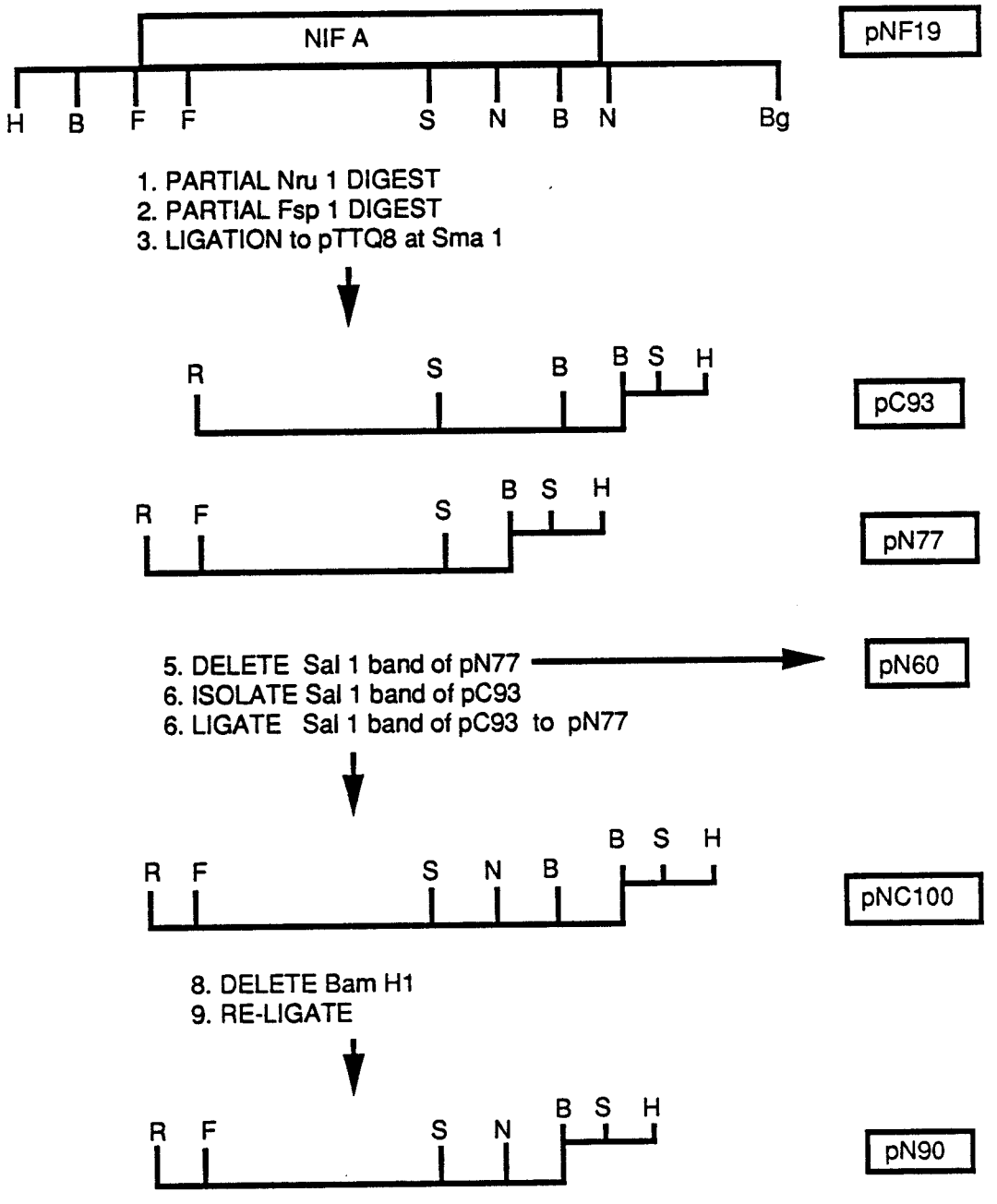
1. Construction of pN77 and pC93. To clone the coding region of the *nifA* gene, the published sequence of NifA was inspected to locate a restriction site as close as possible to the amino-terminal of the predicted protein sequence. An *Fsp*1 restriction site was located, beginning at the 'T" of the predicted initiator codon 'ATG'. This blunt-ended restriction site was predicted to be in the same reading frame as the initiating codon of the pTTQ8 plasmid, if fused at the *Sma*1 site of the plasmid. 21bp of DNA downstream of the predicted carboxy-terminal of the NifA protein is an *Nru* 1 restriction site. This restriction site was used; it is outside the coding region and has a blunt end that can will ligate to the *Sma* 1 site in the TTQ8 vector.

The 2.9 Kb *Hin* DIII -*Eco* R1 restiction fragment of pNF19 was isolated from low gelling temperature agar and partially digested with *Nru* 1. The entire digestion was again electrophoresed through low gelling temperature, and the two *Nru* 1 fragments were isolated. These two bands were partially digested with *Fsp* 1, purified, and ligated to pTTQ 8 digested with *Sma* 1(Figure 3). The ligation mixture was used to transform *E. coli* DH5α and the ampicillin resistant transformants were then analysed by restriction enzyme digestion.

Figure 3.

Construction of NifA expression vectors. The major cloning steps for the construction of pTTQ8 derived expression vectors are shown. The boxed region labeled NIF A is the predicted coding region for NifA. Partial digestion of the pNF19 insert containing the coding region was used to isolate recombinant plasmids pC93 and pN77 after ligation to pTTQ8 linearized at *Sma* 1. Insertion of the *Sal* I restriction fragment from pC93 (intact C-terminal) to pN77 (intact N-terminal) regenerated the desired full-length clone, pNC100. Deletion of the *Sal* I fragment of pN77 gave pN60 directly; similarly, deletion of the *Bam* HI restriction fragment of pNC100 yielded pN90. Restriction enzyme sites are: B, *Bam* HI; Bg, *Bgl* II; F, *Fsp* 1; H, *Hin* DIII; N, *Nru* 1; R, *Eco* R1; S, *Sal* 1. Drawing scale is approximate.

Figure 3


NIF A
pNF19
H B F F S N B N Bg
1. PARTIAL Nru 1 DIGEST
2. PARTIAL Fsp 1 DIGEST
3. LIGATION to pTTQ8 at Sma 1
R S B B S H
pC93
R F S B S H
pN77
5. DELETE Sal 1 band of pN77
6. ISOLATE Sal 1 band of pC93
6. LIGATE Sal 1 band of pC93 to pN77
pN60
R F S N B B S H
pNC100
8. DELETE Bam H1
9. RE-LIGATE
R F S N B S H
pN90

No clone was found to contain the entire 1646 bp *Fsp* 1 - *Nru* 1 insert. However, two clones contained plasmids with overlapping segments of the desired insert. One clone contained a 1246 bp insert from the 5' *Fsp*1 site to the 5' *Nru*1 site; it was purified and designated pN77 (Figure 3). The other clone contained a 1532 bp insert from the 3' *Fsp*1 site to the 3' *Nru*1 site; it was purified and designated pC93 (Figure 3).

2. Constuction of pNC100. The entire coding region of NifA was reconstructed from pN77 and pC93 by resticting pN77 with *Sal* 1and purifying the vector plus attached 990 bp remaining of the original insert, and inserting a 665 bp *Sal* 1 fragment of pC93. (The second *Sal* 1 site derives from the vector polylinker region). Restiction analysis of the ampicillin resistant transformants was used to isolate the desired1646 bp insert expected of a full-length NifA clone. This construct has a 2 amino acid addition immediately following the intiating methionine codon (w.t. = Met-arg-lys; pNC100 = Met-(asn-ser)-lys. From codon three (lys) of the predicted reading frame of pNC100 to the termination codon is wild-type sequence of NifA.

3. Construction of pN90. Plasmid pN90 was constructed by deleting the 212 bp *Bam* H1 fragment of pNC100 and re-circularizing the plasmid by ligation. This deletion places the NifA coding region in frame with the α-peptide of *lacZ* in TTQ8. Accordingly, clones containing the pN90 plasmid exhibit α-complementation and are flourescent on MUG plates. In addition, the final length of the protein is increased by the length of the α-peptide (approximately 100 amino acids).

4. Construction of pN60. Plasmid pN60 was constructed by deleting the 665 bp *Sal* 1 fragment of pNC100 and re-circularizing the the plasmid by ligation. This deletion places the NifA coding region in frame with the α-peptide of pTTQ8; colonies containing the pN60 plasmid are flourescent on MUG plates. In addition, the final length protein is increased by the length of the α-peptide (~100 amino acids).

Construction of recombinant *nif::lac* λ phage

Recombinant λ phages were created by *in vivo* homologous recombination between the plasmids pBZ550, pAZ550, pRAZ550 or pABZ550 and the lambda phage λRS45 (Simons *et al.* 1987). The promoter fusion on the plasmid is transferred to λ phage by homologous recombination, the recombinant phage is isolated, purified, and then used to lysogenize the desired *E. coli* strain. This method of construction obviates the need to ligate DNA directly to λ DNA, and then package the DNA in empty λ phage virions.

Each of the plasmid fusions were transformed (separately) to strain P90C, and verified by plasmid purification and restriction digestion. A culture of each strain was grown overnight, centrifuged and resuspended in λdil buffer (20 mM MOPS, 10 mM MgSO4, 0.01% gelatin, pH 7.2) at twice the initial cell density. To 0.1mL of cells was added ~10^7 - 10^8 λRS45 phage, and the mixture was incubated on ice for 20 minutes. After incubation, 10 mls of λLB was inoculated with the infected cells and the cultures were grown to stationary phase or until lysis occured. The cultures were sterilized with chloroform, and centrifuged. The supernatants (phage lysates) should contain approximately 1/100,000 recombinant phage

(Simons *et al.* 1987). The lysates were then used to re-infect a fresh culture of P90C (as described above) and grown for 1 hour to express kanamycin resistance. The cultures were centrifuged, the cell pellets washed in LB, re-centrifuged, and the cells plated on kanamycin/Xgal LB media and incubated at 30 °C. Lac^+, kan^r colonies were collected and tested for ampicillin sensitivity, to insure the sterilized phage lysates did not contain surviving cells with the ampicillin and kanamycin resistant plasmid.

Colonies of the proper phenotypes were then grown overnight in λLB and the culture supernatants were then plated on a lawn of P90C and Xgal. The plaques arising from this infection are phage spontaneously released from the *recA*+ P90C strain. The culture supernatants of the lysogenic cultures produced some lac-negative ("white") plaques; these are contaminating non-recombinant phage from the original lystae. A single blue plaque from each plate was then plaqued twice in the same manner to purify the recombinant phage. A high-titer lysate of each fusion was then prepared and stored.

The purified recombinant *nif::lac* λ phage were used to lysogenize *E. coli* PE56. Plasmids pNF19, pCH2 (Hertig *et al.* 1989), pNC100, pN90, pN77, pN60, pC93, and pTTQ8 were used to transform PE56, and the transformants verified by plasmid purification and restriction analysis. Overnight cultures of PE56 in SOB containing the desired plasmid, and a plasmid-less control, were centrifuged and resuspended in λdil as described above, then infected at a multiplicity of infection of 0.001. This phage/cell ratio insures that no di-lysogens will form from multiple infection. The phage/cell mixture was kept on ice for 20 minutes, and grown aerobically at 30 °C. in LB media

for one hour to express kanamycin resistance. The culture was then plated on LB media with kanamycin, Xgal, and ampicillin (except the plasmid-less control for which amp was omitted). The resulting lysogenic colonies were then streak purified three times and stored at -70 °C.

β-Galactosidase Enzyme Assays.

Enzyme assays for β-galactosidase (reported as "lac activity") were done according to Miller (Miller 1972), with the following modifications. Z buffer was prepared with a two-fold higher concentration of β-mercaptoethanol. Exactly 0.5 mL of Z buffer was used per assay. The added volume of culture varied, but was made up to 0.5 mL with CAM media if less than 0.5 mL of cells were added. This method gave a uniform concentration of β-mercaptoethanol per assay and a constant ratio of Z buffer to media, in contrast to Miller's procedure, in which the ratio varies. The optical density (OD) of the cultures was measured at 550 nm, not 600 nm as given by Miller. Optical density measurements at 550 nm were found to give a more linear relationship between cell density and optical density as cell density increased (data not shown); when corrected for non-linearity, cultures measured at OD_{600} had identical *lac* activity as those cultures measured at OD_{550}.

The enzyme activity reported for all assays (see Results) is in "Miller units". Miller units of β-galactosidase activty are arbitrary, but are proportional to the β–galactosidase enzyme concentration per cell; the unit is defined so that wild-type *E. coli* cultures have approximately 1000 units of activity in a fully induced culture grown in minimal glucose media. In the

galactosidase, based on a calculation given by Miller (Miller 1972). I chose not to do so, as the specific activity calculation is only applicable when the protein content of the cells, as percent of cell dry weight, is known. The experiments described here (see Results) were conducted with a large variety of aerobic and anaerobic physiological conditions. Because I did not specifically verify a uniform content of cellular protein under all physiological conditions, specific activty units would be unsupportable.

RESULTS

Results are presented in two sections. In the first section, **Results A. The *nifB* promoter**, the activation of a *nifB::lac* fusion by NifA is described. These experiments define the physiological conditions necessary for transcriptional activation of *nifB* by NifA constitutively expressed from a plasmid.

The second section, **Results B. The *nifA* promoter**, analyses the physiological conditions necessary for activation of the *nifA* promoter when stimulated by a constitutively expressed FixJ protein. Included in this section is an analysis of the reconstructed regulatory cascade with constitutively expressed FixJ stimulating a single, lysogenic copy of the *nifA* gene, producing wild-type NifA protein that then activates the *nifB* fusion.

RESULTS A. The *nifB* promoter

Characteristics of the *nifB*::lac and *nifA*::lac operon fusion strains.

The introduction of the recombinant phage λBZ45, or λABZ45, (Table 1) both containing an operon fusion of the *nifB* promoter, to *E. coli* PE56 resulted in the isolation of kanamycin resistant colonies. Infection of a culture of these cells by the hetero-immune phage λNK395 (Simons *et al.* 1987) released phage that produced blue plaques in the presence of Xgal, and no plaques when infected with a homo-immune phage λNK330. Phage isolated from the blue plaques transduced kanamycin resistance and the blue-plaque phenotype to a fresh culture of PE56. These results demonstrated the kanamycin resistance and *lac*$^+$ phenotype of the transductant colonies was due to lysogenization by the recombinant phage λBZ45 or λABZ45.

When compared to the control (strain PE5645, Table 1), BZ45 or ABZ45 colonies on Xgal plates contained faintly blue centers after prolonged incubation. PE5645 colonies were slightly darker and more uniform blue than BZ45. These intial observations suggested that the inclusion of the *nifB* region in to the *lacZYA* operon reduces background expression of *lac* activity. Simons has noted that the inclusion of almost any cloned fragment into the λ(*lac*) vectors will cause an increase in *lac* expression (Simons *et al.* 1987). However, the region immediately preceding the *nifB* promoter is speculated to be the transcriptional terminator region for the *nifA* gene (Weber *et al.* 1985). It is likely that BZ45 and ABZ45, constructed

as promoter fusions, also contain a transcriptional termination signal for NifA that reduces transcription through the *nifB::lac* operon fusion. The expression of ABZ45 was not different than BZ45, and demonstrated that although ABZ45 contains a wild-type promoter and coding sequence for NifA, there is no activation of the *nifB* promoter fusion immediately downstream without prior activation of the *nifA* promoter (see Results B. below).

Table 2 contains a quantitative analysis of the *nifB*::lac fusion (strain BZ45), the *nifA-nifB::lac* fusion (strain ABZ45), the *nifA*::*lac* fusion (strain AZ45), and the reverse promoter fusion (strain RAZ45) with and without plasmids pNF19 and pCH2. Under aerobic conditions, PE5645 had 4-fold higher lac activiy than BZ45, confirming the effect noted on Xgal plates (above). Anaerobic conditions had no effect on the control, while BZ45 was stimulated about four-fold. This activation must be have due to non-NifA stimulated transcription of the *nifB* promoter. The addition of pNF19, constitutively expressing NifA from the *lac* promoter of pUC19, caused a 250-fold increase in the *lac* activity of the *nifB* promoter. The addition of the FixJ plasmid pCH2, constitutively expressing FixJ from the *lac* promoter of pUC19, had no effect aerobically or anaerobically. This result provided two controls: *(i)* a specific *R. meliloti* transcriptional activator protein (NifA) was required to activate the *nifB* promoter and a different activator protein from *R. meliloti* (FixJ) would not suffice, and *(ii)* the stimulation by pNF19 cannot be due to the pUC19 vector, which is common to both pCH2 and pNF19.

The addtion of FixJ (pCH2) to AZ45 stimulates a ~100 fold increase in activity anaerobically, but has no effect aerobically. This result is

Table 2

Aerobic and anaerobic expression of *nifB*, *nifA*, *rev*A, and *nifA-nifB* fusions. An overnight stationary phase aerobic culture of each strain in CAM was diluted 1:100 intoCAM-xylose media and grown to an OD_{550} ~2.0. The cultures were diluted 1:50 into the same media and made anaerobic by the addition of Oxyrase. Lac activity (Miller units) was monitored throughout the growth of the culture and the reported value is that determined when the culture entered stationary growth. 1:100 dilutions of the overnight cultures were diluted into CAM-xylose media and maintained in exponential aerobic growth at low cell densities (OD_{550} < 0.2) by serial 1:5 dilutions into pre-warmed media for at least five generations before harvest (see Materials and Methods for harvesting details) and the *lac* activity was determined. The genotypes of the strains are given in Table 1.

Table 2

		LAC ACTIVITY	
Strain	Plasmid	Aerobic	Anaerobic
PE5645	-	2	2
PE5645	pNF19	2	2
PE5645	pCH2	2	2
BZ45	-	.5	2
BZ45	pNF19	2	500
BZ45	pCH2	.5	2
AZ45	-	1	1
AZ45	pCH2	1	125
AZ45	pNF19	1	1
ABZ45	-	2	2
ABZ45	pCH2	2	25
ABZ45	pNF19	2	500
RAZ45	-	25	45
RAZ45	pNF19	25	45
RAZ45	pCH2	25	30

different than Dr. David Kahn's report (from whom pCH2 was a gift) , *i.e.* - the inclusion of pCH2 to a protein fusion of the *nifA* promoter on a multi-copy plasmid stimulated *lac* activity 200-fold aerobically and 100-fold anaerobically (Hertig *et al.* 1989). There was no stimulation of AZ45 by pNF19, which demonstrated that NifA is not auto-regulatory. Neither pCH2 or pNF19 had an effect aerobically or anaerobically on the control strain PE5645.

pCH2 stimulated the ABZ45 fusion ~12-fold anaerobically, and had no effect aerobically. Note that, in this case, the *nifB* promoter forms the fusion and the NifA protein is derived from FixJ stimulation of the (lysogenic) wild-type *nifA* gene. The 12-fold stimulation of *nifB* by NifA derived from the single-copy gene was 20-fold less than the ~250-fold stimulation that was observed when NifA was constitutively expressed from the pUC19 *lac* promoter. The addition of pNF19 to ABZ45 stimulated the *nifB* promoter 250-fold, the same as it did for the BZ45 construct. This result indicated that the *nifB::lac* fusion was intact in the ABZ45 construct and that the low activity of pCH2-ABZ45 may have been due to different NifA concentrations in the cell when *nifA* is transcribed from a single-copy lysogen or from a multi-copy plasmid.

The RAZ45 strain expressed 25-fold higher activity aerobically than does the AZ45/pCH2 strain and confirmed the effect noted on Xgal plates. There was less than a 2-fold stimulation of RAZ45 when grown anaerobically. NifA (pNF19) did not effect the *revA* promoter fusion aerobically or anaerobically, but FixJ (pCH2) reduced the *revA::lac* activity anaerobically. the anaerobic decrease may have been due to the 100-fold anaerobic

opposing stimulation of the *nifA* promoter. One final point is appropriate; FixJ stimulated 125 units of activity from the *nifA* promoter (as measured by AZ45/pCH2), but only one-fifth the activity (25 units) was detected in strain ABZ45. In other words, 125 units of activity reached the end of the NifA coding region, but only 25 units appeared on the distal side of the *nifB* promoter, even though wild-type NifA protein was presumably produced to activate the *nifB* promoter. This result is evidence for the proposed transcriptional terminator (of the *nifA* transcript) between the *nifA* and *nifB* genes (see above).

The data presented in Table 2 is the basis for the physiological analysis of the *nifA* and *nifB* promoters. When considered together, these data support the conclusion that the lysogenic operon fusions are a sensitive system for the analysis of aerobic and anaerobic control of *Rhizobium meliloti* nitrogen fixation gene expression.

Media effects on *nifB* expression.

The 250-fold stimulation of activity noted for BZ45/pNF19 (Table 2) under anaerobic conditions was determined after fermentative growth in CAM-xylose media. However, the selection of xylose fermentation as the relevent growth condition involved extensive experimentation and will be described in detail below.

NifA stimulated expression of the *nifB* operon fusion (BZ45/pNF19) was determined during anaerobic fermentation and anaerobic nitrate respiration [I define anaerobic fermentation to be the growth of a culture under strict anaerobic conditions such that energy (ATP) is generated solely

through substrate-level phosphorylation; anaerobic respiration is defined to be the addtion of a alternative electron acceptor, capable of supporting growth on a non-fermentable carbon source, to an anaerobic culture. Energy (ATP) can be generated during anaerobic respiration by substrate-level phosphorylation or by electron transport to a non-oxygen electron acceptor through a terminal reductase enzyme and oxidative phosphorylation]. Lac activity was found to vary ~10-fold (Figure 4) during the fermentation of different carbon sources.

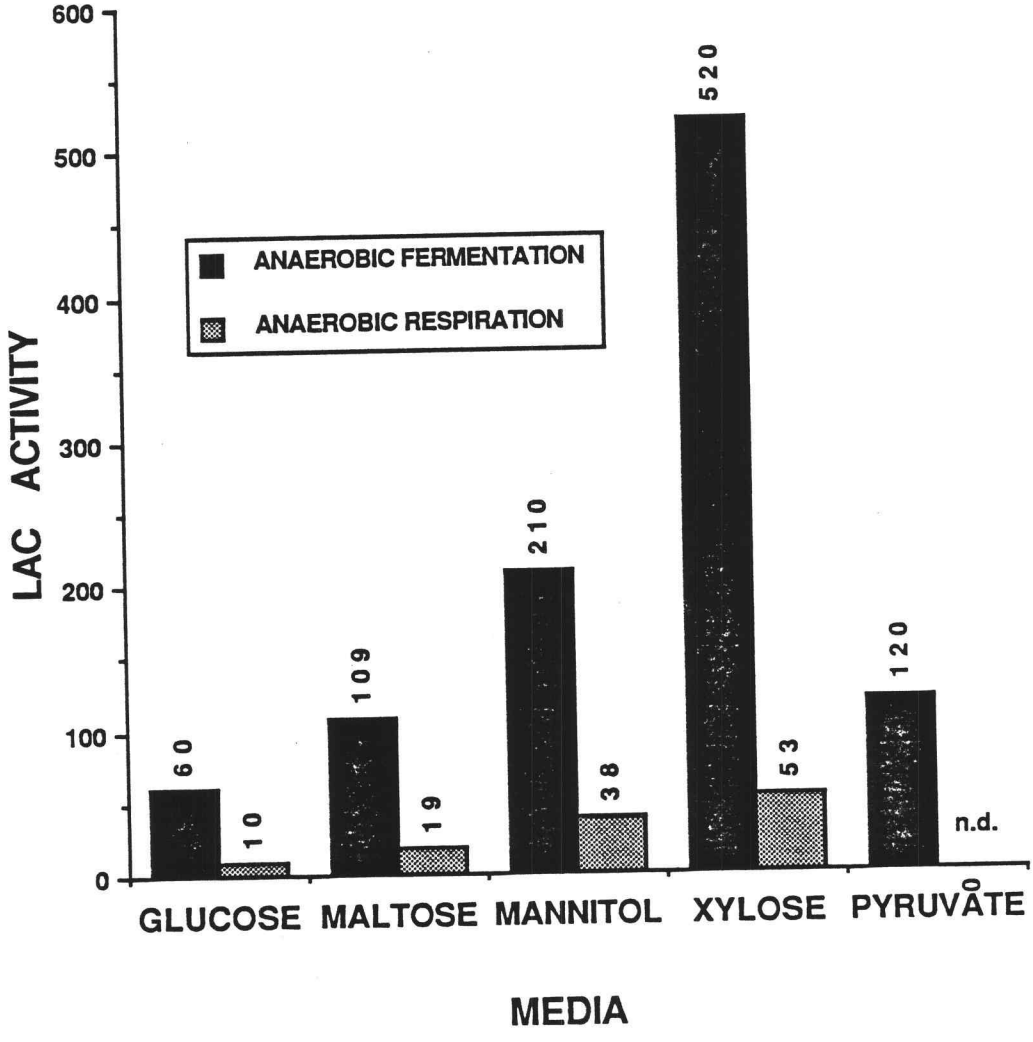
The low value found for glucose fermentation was not due to cyclic-AMP (cAMP) mediated catabolite repression. The addition of either 0.5mM or 5mM cAMP did not alter the glucose value (data not shown).Further, the addition of 5mM cAMP to maltose media did not change the 5-fold difference between maltose and xylose. Maltose is not a catabolite-repressing sugar, i.e. - it does not exhibit diauxic growth in the presence of xylose (Monod 1942). Together, these results support the conclusion that the carbon source effect was not due to cAMP mediated catabolite repression.

E. coli can grow fermentatively with pyruvate as the sole carbon source (for a review, see Knappe 1987). Because no redox chemistry is involved in the anaerobic catabolism of pyruvate (no NADH is generated), the relatively oxidized three-carbon compound pyruvate can support fermentation, while the more reduced three-carbon polyol glycerol cannot (see Clark 1989 for a discussion of this point). Cultures fermenting pyruvate had *lac* activities similar to cultures fermenting maltose (~100 units), although these two carbon sources enter glycolysis essentially at either "end" of the glycolytic pathway. This result suggested that factors other than

Figure 4

Carbon source effects on *nifB* expression. A stationary phase overnight culture of BZ45/pNF19 in CAM was diluted 1:100 into CAM and grown aerobically to an OD_{550} of ~2.0, then diluted 1:50 into anaerobic media containing different carbon sources (ANAEROBIC FERMENTATION). Identical cultures were supplemented with nitrate (ANAEROBIC RESPIRATION). *Lac* activity (Miller units) was measured throughout the growth of each culture; the reported value is when the culture entered stationary growth. No pyruvate-nitrate culture was grown (n.d = not done).

Figure 4


600
500
400
300
200
100
0
LAC ACTIVITY
ANAEROBIC FERMENTATION
ANAEROBIC RESPIRATION
60
10
109
19
210
38
520
53
120
n.d.
GLUCOSE
MALTOSE
MANNITOL
XYLOSE
PYRUVATE
MEDIA

the mixed-acid regeneration of NAD^+ are responsible for the variation in *lac* activity observed with different carbon sources.

The addition of nitrate to all fermentable sugars caused a carbon-source dependent reduction in *lac* activity (Figure 4). While nitrate reduced the glucose fermentative value ~4-fold, it reduced the xylose value about 10-fold. The repressive effect of nitrate respiration must therefore be associated with carbon catabolism. The wide range of activity found during fermentation and nitrate respiration with different carbon sources indicates that careful control of physiological variables is important for analysis of *nif* promoter fusions in *E. coli*.

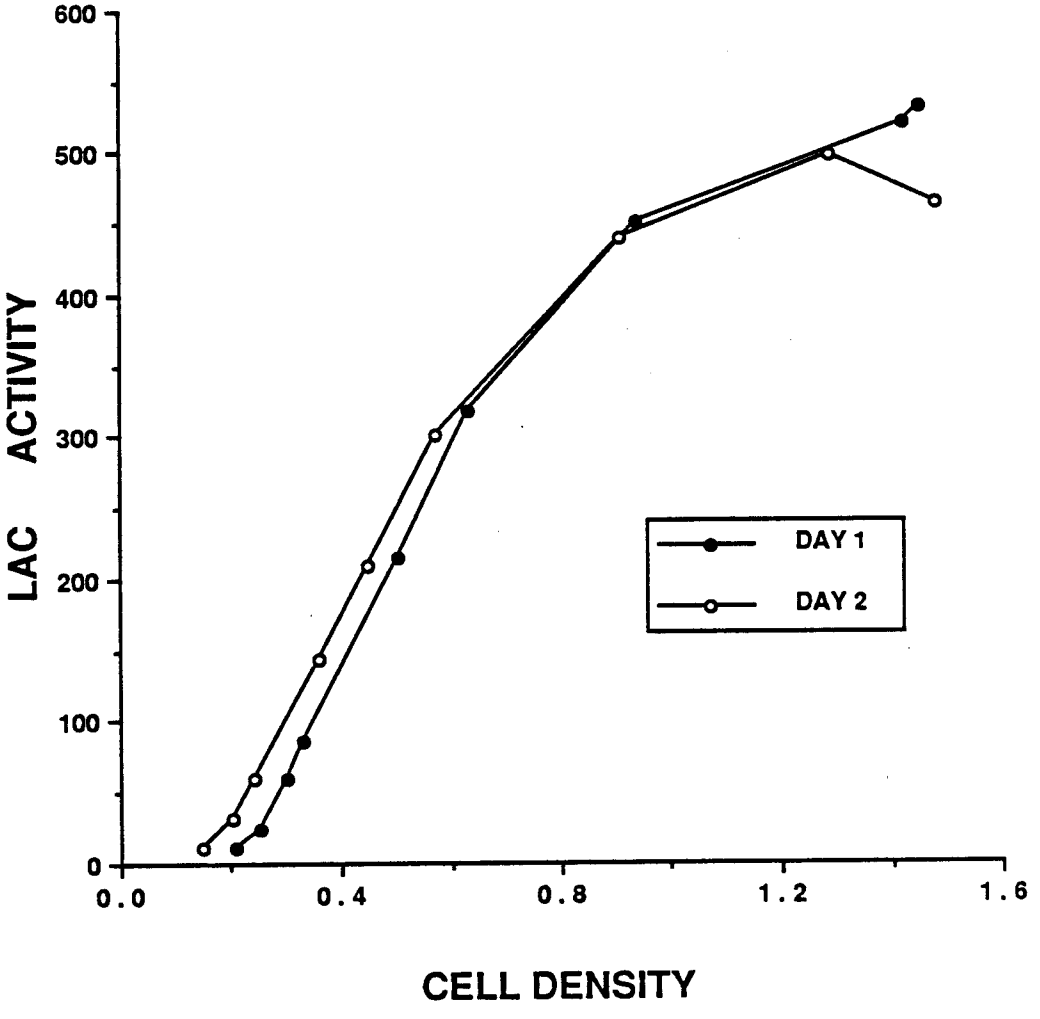
Effect of growth conditions on *nifB* expression

The initial measurements of *lac* activity for BZ45/pNF19 cultures were not reproducible on separate days. The variation in activity occured with both fermentative and nitrate respiration cultures, and was as much as three-fold. Therefore, the lac activity was determined for all anaerobic cultures throughout growth of the culture to stationary phase. Figure 5 shows the induction kinetics of BZ45/pNF19 cultures on separate days. Aerobic cultures were maintained in exponential phase for at least five generations in xylose media, then made anaerobic by the addition of Oxyrase. The *lac* activity was then monitored during the entire growth phase of the culture. The *lac* activity measured at each culture density increased continuously during growth, and did not reach a stable value before stationary phase was attained. Both the rate of induction and the

Figure 5

Reproducibility of the induction kinetics of *nifB* expression. An overnight culture of BZ45/pNF19 in CAM media was diluted 1:100 into CAM-xylose media and maintained in exponential aerobic growth for at least five generations at low cell density (OD_{550} <0.2) by serial 1:5 dilutions into pre-warmed media. The final dilution (OD_{550} of ~0.05) of the culture was made anaerobic by the addition of Oxyrase and the lac activity (Miller units) was determined throughout the growth of the culture. The induction kinetics on two separate days is shown. Cell density is OD_{550}.

Figure 5


600
500
400
300
200
100
0
LAC ACTIVITY
0.0
0.4
0.8
1.2
1.6
CELL DENSITY
DAY 1
DAY 2

final value were very similar on separate days, indicating that it was possible to reproduce the induction kinetics. The initial irreproducible results were primarily due to sampling the cultures at a single time point after induction, while the culture was undergoing continued induction of *nifB* expression (Figure 5).

Effects of innoculum on induction kinetics.

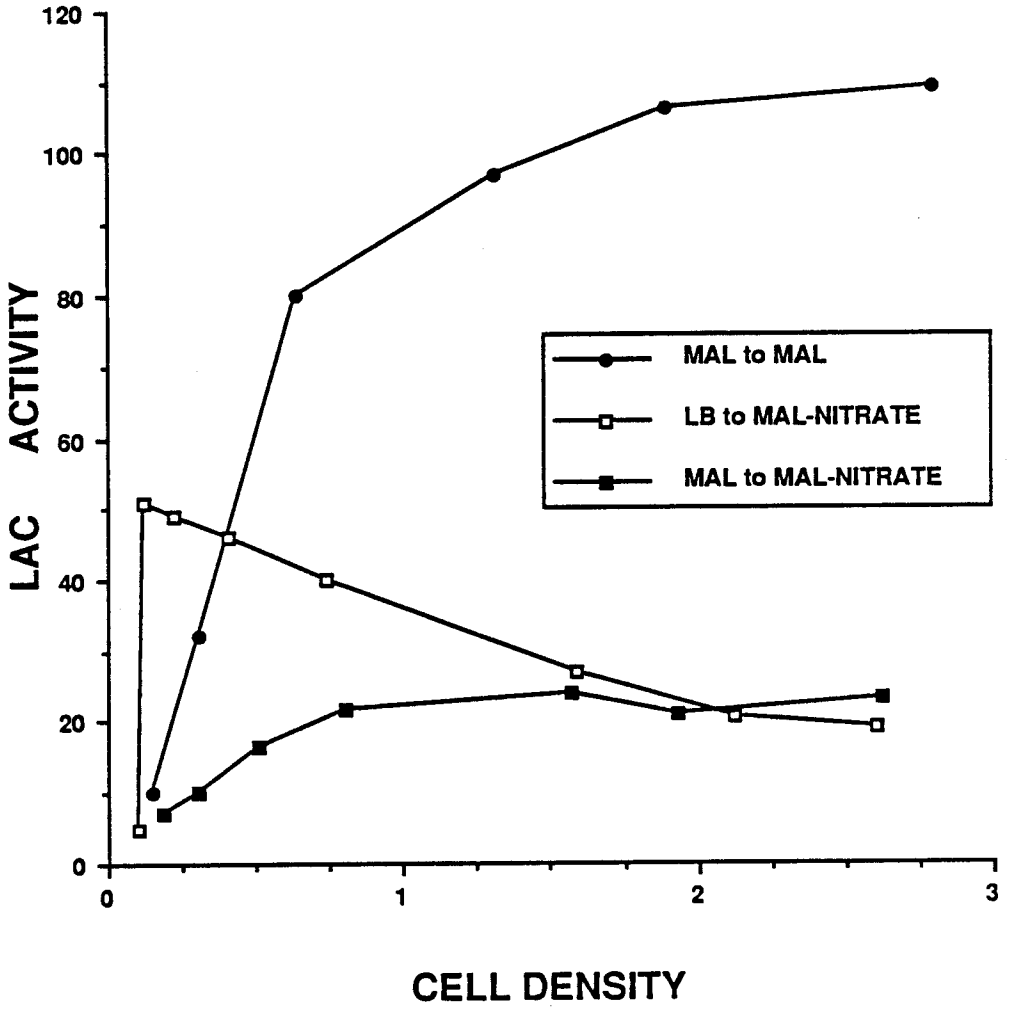
As noted for the results shown in Figure 5, a reproducible induced level of *lac* activity for the *nifB* fusion could only be determined when special care was taken to reproduce the shift from aerobic to anaerobic conditions and the lac activity was monitored throughout the growth of the culture. Figure 6 presents the data for other factors that were found to influence the analysis of lac activity from the *nifB* fusion.

When an aerobic culture growing exponentially in maltose was made anaerobic by the addition of Oxyrase, the lac activity increased continuously to the final value (Figure 6), as occurs with xylose cultures (Figure 5). When an identical culture was supplemented with nitrate just prior to addition of the Oxyrase, the lac activity increased with different kinetics to a final stable value, that was 5-fold lower than the fermentative culture (see also Figure 4). When an overnight (stationary phase) culture grown in Luria broth was diluted 1:100 into anaerobic maltose-nitrate media, a rapid and transient increase in lac activity occured, followed by a a steady decline in the lac activity to a final value, essentially as occurred in the maltose to maltose-nitrate culture. This result (together with the data presented in Figures 4 and 5), defined three experimental conditions required to repro-

Figure 6

Inoculum effects on *nifB* expression. A stationary phase aerobic overnight culture of BZ45/pNF19 in Luria broth was diluted 1:200 into anaerobic CAM maltose-nitrate media (LB to MAL-NITRATE). A separate culture was grown aerobically overnight in CAM, diluted 1:100 into CAM-maltose, and maintained in exponential growth for at least five generations at low densitiy (OD_{550} <0.2) by serial 1:5 dilutions into pre-warmed media before addition of Oxyrase (MAL to MAL). Nitrate was added to half of the maltose culture before addition of the Oxyrase (MAL to MAL-NITRATE). The lac activity (Miller units) was determined throughout the growth of each culture to stationary phase. Cell density is OD_{550}.

Figure 6


120
100
80
60
40
20
0
LAC ACTIVITY
0
1
2
3
CELL DENSITY
MAL to MAL
LB to MAL-NITRATE
MAL to MAL-NITRATE

ducibly measure lac activity of the *nifB* promoter. A defined carbon source, constant monitoring of the induction of lac activity, and a defined method of inoculation from an exponentially growing aerobic culture are all critical for a reproducible analysis. A single analysis, based on the dilution of an overnight culture (grown in undefined media) to anaerobic conditions and then determining the lac activity at an arbitrary time after shift to anaerobic conditions, leads to entirely different conclusions about the induction of *nifB* activity, than an analysis based on cultures grown under more defined conditions.

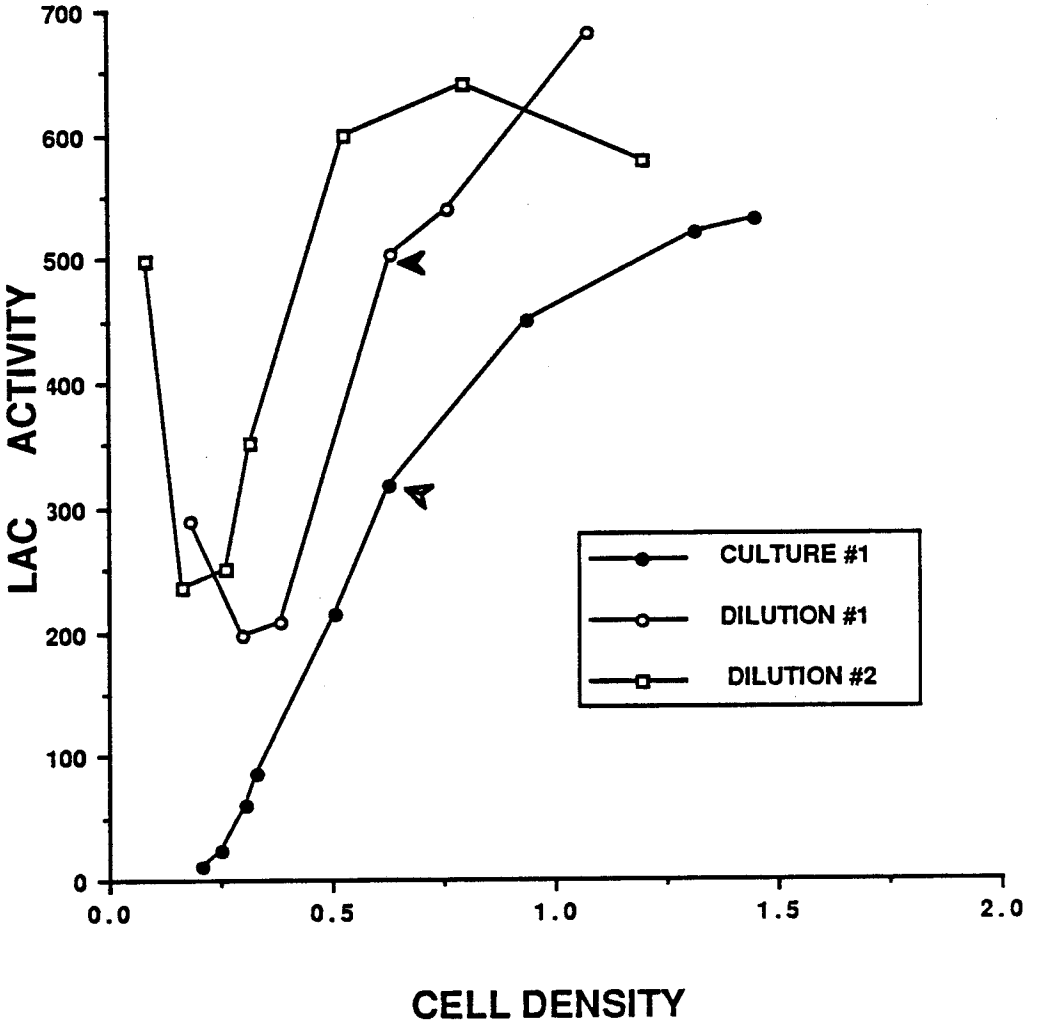
Balanced anaerobic growth and induction kinetics.

If the physiological state of a culture prior to transfer from aerobic to anaerobic conditions is a critical factor for determining the *lac* activity of the *nifB*::*lac* fusion (Figures 5 and 6), then (by inference) the physiological state of the cells after transfer may similarly be important. Neidhardt notes that *E. coli* can be maintained in balanced anaerobic growth (Smith and Neidhardt 1983), just as it can be maintained for aerobic cultures (the guiding principle of the chemostat). It was therefore relevent to try and attain balanced anaerobic growth of a cuture, by serial dilution between anaerobic media, and analyse the effects on *nifB* induction. Theoretically, it should be possible to define the anaerobic conditions in which the *nifB* gene is transcribed at a constant rate and thereby producing a constant level of lac activity proportional to the number of cells in culture (assuming no other special effects on induction other than anaerobiosis are required for induction).

Figure 7

Expression of *nifB* activity after anaerobic dilution by serial transfer. A stationary phase aerobic overnight culture of BZ45/pNF19 in CAM was diluted 1:100 into CAM-xylose media and maintained in exponential aerobic growth for at least five generations at low cell density (OD_{550} <0.2) before the addition of Oxyrase. When the cell density was ~0.6 (CULTURE #1), a portion was transferred anaerobically to a fresh tube of anaerobic media (open arrow, DILUTION #1). When the cell density of DILUTION #1 reached OD_{550} ~0.6, the culture was again transferred anaerobically to fresh anaerobic media (closed arrow, DILUTION #2). The lac activity (Miller units) was determined throughout the growth of each culture to stationary phase. Cell density is OD_{550}.

Figure 7


700
600
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
LAC ACTIVITY
CELL DENSITY
CULTURE #1
DILUTION #1
DILUTION #2

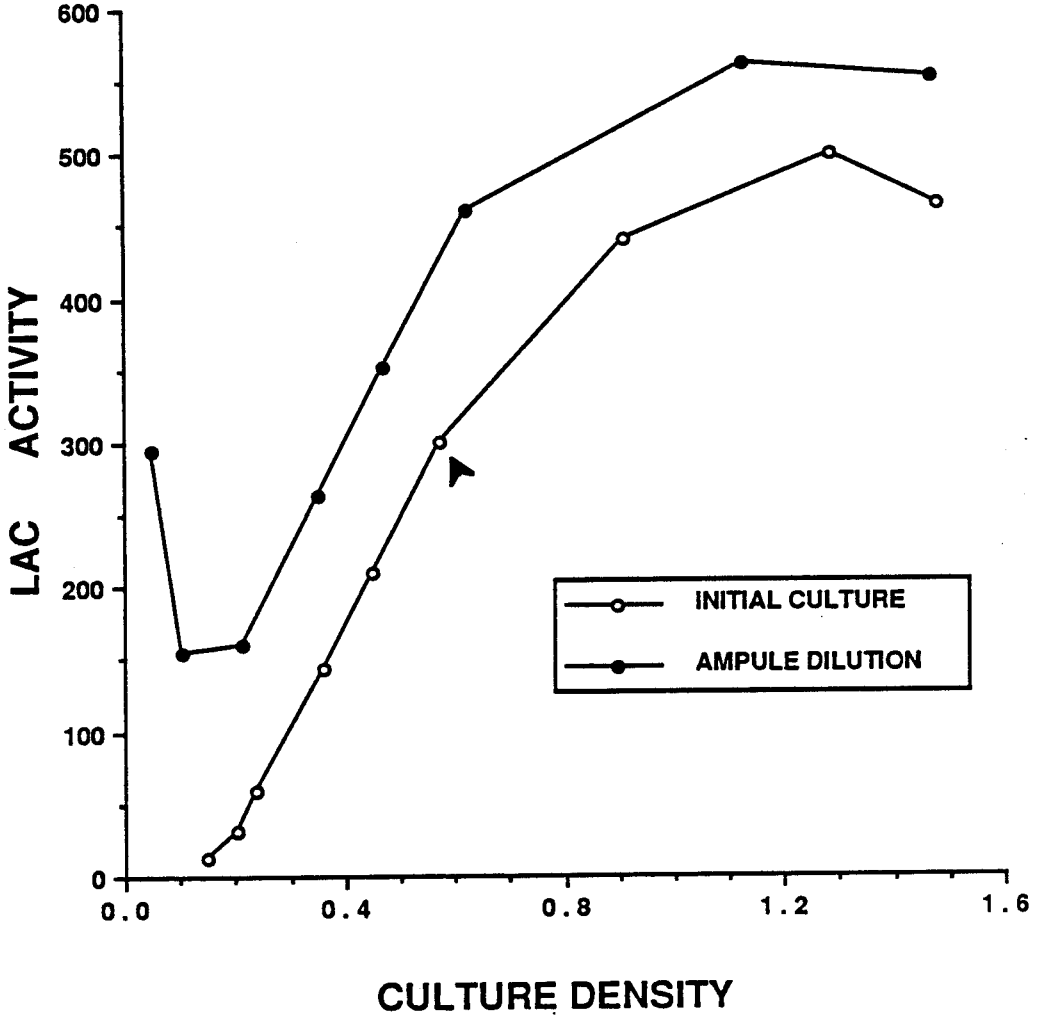
When an exponentially growing anaerobic xylose culture was diluted into pre-warmed anaerobic media, an immediate (but transient) decay in the lac activity occured (dilution #1, Figure 7), after which the lac activity again increased continuously. A second dilution had the same effect (dilution #2, Figure 7). As the lac activity decreased ~50% when the culture density doubled, the decay in activity can be attributed to simple dilution if *nifB* expression was transiently turned "off". The dilutions described above were made by pipet transfer between a growing anaerobic culture and a fresh tube of anaerobic media by sealing the cells to be transferred between two layers of paraffin oil in a Pasteur pipet during transfer to the fresh media. This method should have prevented oxygen contamination. Extreme care was taken to insure that the transferred cells were not exposed to oxygen. Nontheless, the lac activity declined rapidly after transfer. Although the growth rate of the initial culture and the diluted cultures was stable for at least eight generations (data not shown) and the cultures appeared to have attained "balanced" anaerobic growth (Smith and Neidhardt 1983), a stable induced rate of *nifB* expression could not be maintained. Therefore, an experiment was devised so that an anaerobic culture could be diluted without transfer of the cells.

To dilute an anaerobic culture of BZ45/pNF19 without transfer of the cells, a exponentially growing aerobic culture was made anaerobic by the addition of Oxyrase and a portion was flame-sealed in a very thin glass ampule blown from a Pasteur pipet. The ampule was placed in the bottom of a culture tube and the tube was filled with media and made anaerobic with Oxyrase. A magnet was taped to the outside of the tube and a sterile

Figure 8

Expression of *nifB* activity after anaerobic dilution without transfer. A stationary phase aerobic overnight culture of BZ/NF19 in CAM was diluted 1:100 into CAM-xylose media and maintained in exponential aerobic growth for at least five generations at low cell density ($OD_{550} < 0.2$) before addition of Oxyrase. The culture was divided between a glass ampule and a separate tube and the lac activity (Miller units) of the tube was monitored (INITIAL CULTURE). The kinetics of lac expression for the ampule culture after anaerobic dilution was also determined (AMPULE DILUTION). See text for details of the anaerobic dilution apparatus. Cell density is OD_{550}.

Figure 8


600
500
400
300
200
100
0
LAC ACTIVITY
0.0
0.4
0.8
1.2
1.6
CULTURE DENSITY
INITIAL CULTURE
AMPULE DILUTION

magnet was placed inside the tube and held in place by the outer magnet. The tube containing the culture ampule and the magnet, and the culture used to fill the ampule were incubated. Samples were withdrawn from the tube culture and lac activity determined. When the lac activity was ~300 units, the external magnet was removed from the ampule-containing culture, which released the interior magnet to fall and crush the ampule. In this manner the culture was diluted without transfer. A 50% decline in lac activity per culture OD occured after dilution, followed by an increase in activity (Figure 8) and confirmed the results shown in Figure 7. A reasonable explanation for the dilution effect (Figures 7 and 8) is that an excreted metabolite produced by the growing culture is required for the induction of the *nifB* promoter, and it is diluted when the culture is diluted. The effect could also be due to a change in physiological state that is not manifested by a change in growth rate. What-ever the explanation, and assuming the ampule dilution experiment was done entirely anaerobically as designed, it is reasonable to conclude that *nifB* expression is subject to a previously unreported control which operates only under anaerobic conditions.

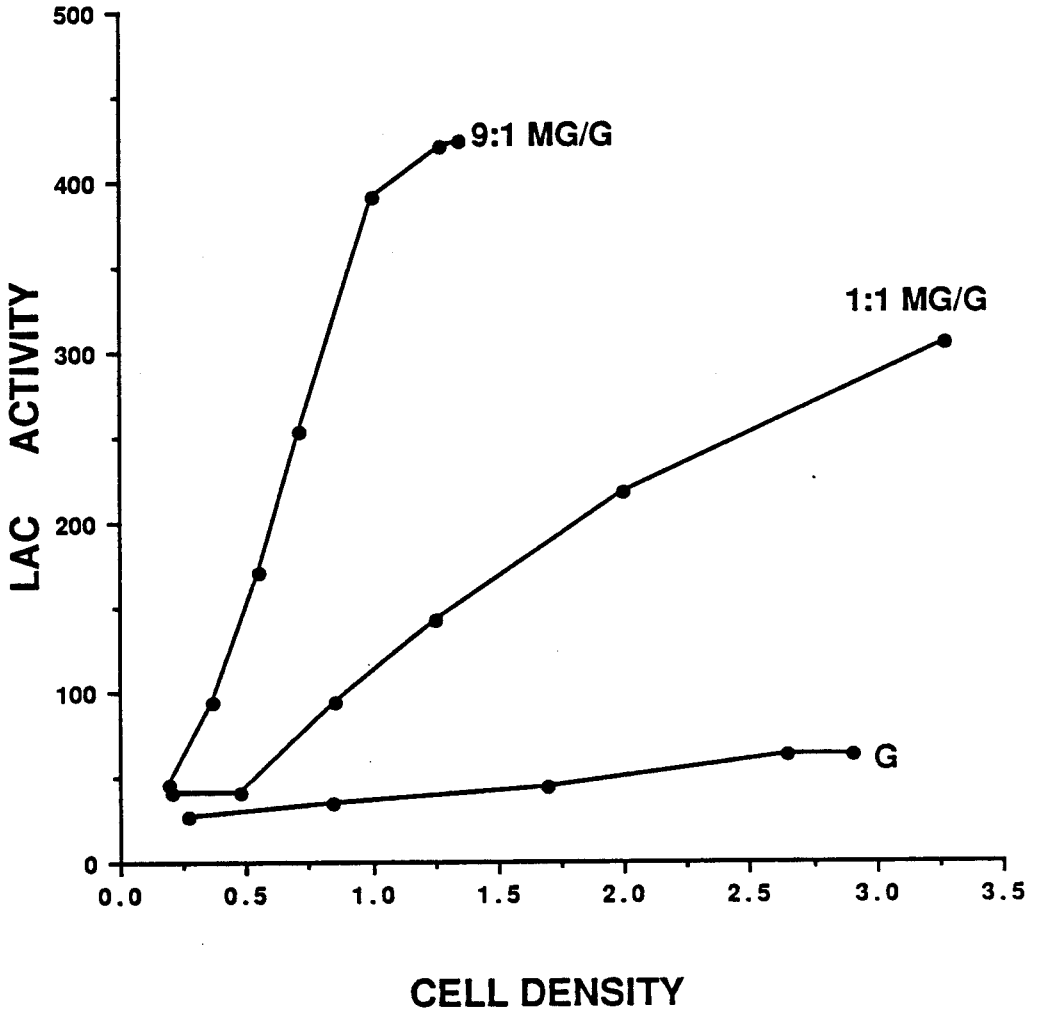
Effects of α-methyl glucose on *nifB* expression in CAM-glucose

As noted previously, each carbon source tested produced a different level of lac activity from the *nifB* operon fusion (Figure 4). The kinetics of induction for the *nifB* promoter when cultures were grown fermentatively in 25 mM glucose is shown in Figure 9. The lac activity slowly increased to ~50 units during the entire growth of the culture. In media with equal

Figure 9

Effect of α-methyl glucose addition on *nifB* expression in glucose cultures. A culture of BZ45/pNF19 was grown aerobically overnight in CAM, then diluted 1:100 in CAM-glucose media and grown aerobically to OD_{550} ~2.0. The late exponential phase glucose culture was then diluted 1:50 in CAM-25mM glucose (G), a 1:1 mixture of CAM glucose/α-methyl glucose (25 mM each) (1:1 MG/G), or a 9:1 mixture of CAM glucose/α-methyl glucose (10 mM glucose and 90 mM α-methyl glucose) (9:1 MG/G). Oxyrase was added to each culture and the lac activity (Miller units) was monitored throughout anaerobic growth of the cultures. Cell density is OD_{550}.

Figure 9


500
400
300
200
100
0
LAC ACTIVITY
9:1 MG/G
1:1 MG/G
G
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
CELL DENSITY

concentrations of α-methyl glucose and glucose, the lac activity increased more than 6-fold. When α-methyl glucose was added in nine-fold excess of glucose, the lac activity increased at an even faster rate to more than 400 units of activity.

Effects of carbon source on nitrate respiration.

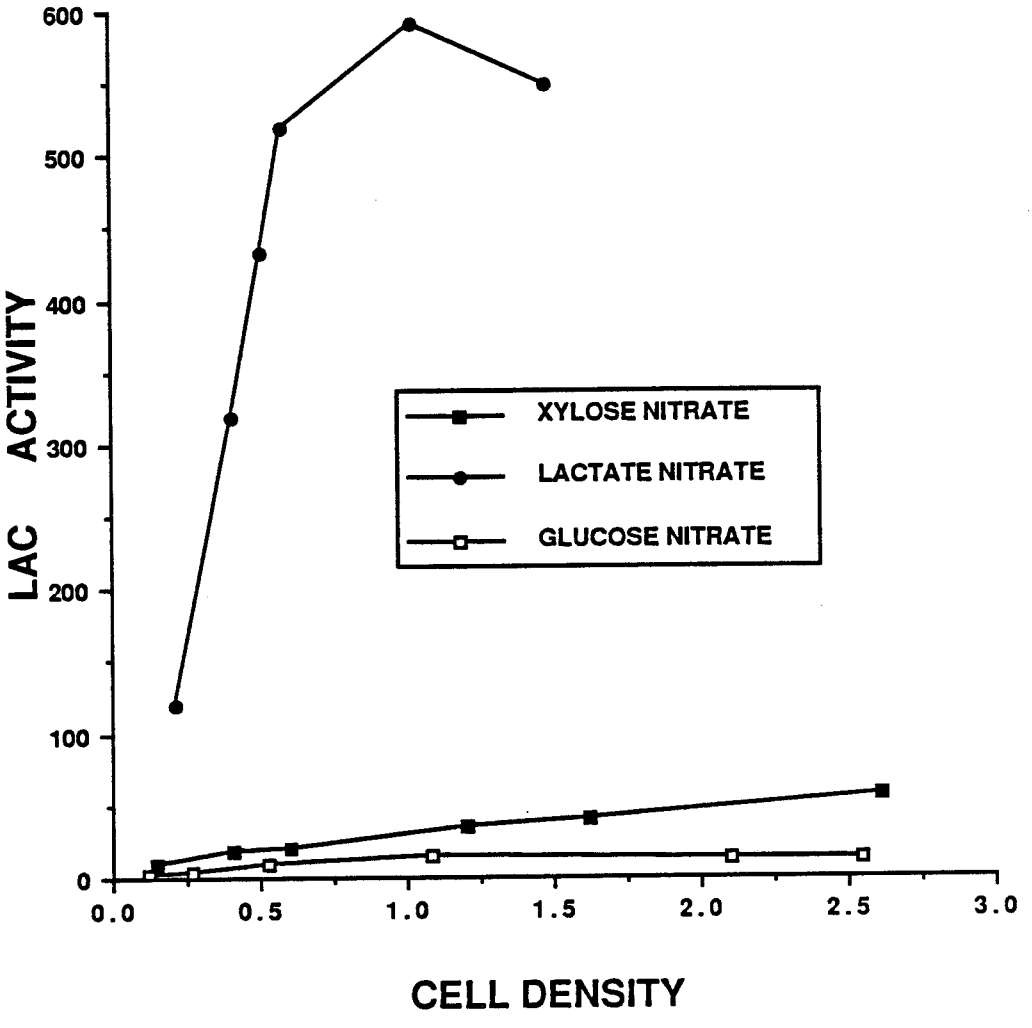
Figure 4 showed that the effect of nitrate respiration varied between carbon sources. Lactate is a non-fermentable carbon source for *E. coli;* by definition, anaerobic growth with lactate as the carbon source requires the addition of a respiratory substrate. Lactate is an electron donor for nitrate reductase (Lin 1987) through two distinct respiration-linked lactate dehydrogenases. The respiration-linked lactate dehydrogenases of *E. coli* produce pyruvate from lactate, with no evidence for a physiological relevant reverse reaction (Lin 1987). The respiratory lactate dehydrogenases are distinct from the fermentative lactate dehyrogenase that converts pyruvate to lactate and regenerates NAD^+ during fermentation (Clark 1989).

In the presence of lactate and nitrate, BZ45/pNF19 cultures produced high levels of lac activity from the lysogenic fusion (Figure 10). In comparison, cultures grown in xylose-nitrate and glucose-nitrate produced ten-fold less activity. The final level of lac activity attained in lactate-nitrate was, once again, only reached after a long period of induction during the entire anaerobic growth phase. The lac activity measured in any carbon source, with or without nitrate, was found to have a characteristic value (Figure 4), and the final activity was attained slowly (Figures 5-10).

Figure 10

Kinetics of *nifB* expression during anaerobic respiration of nitrate. A stationary phase aerobic overnight culture of BZ45/pNF19 in CAM was diluted 1:100 into CAM with different carbon sources and grown aerobically to an OD_{550} of ~2.0. The late exponential phase cultures was then diluted 1:50 into anaerobic CAM-nitrate media containing glucose, xylose, or lactate as the carbon source (GLUCOSE-NITRATE, XYLOSE-NITRATE, or LACTATE NITRATE). The lac activity (Miller units) was monitored throughout the growth of the culture. Cell density is OD_{550}.

Figure 10


600
500
400
300
200
100
0
LAC ACTIVITY
XYLOSE NITRATE
LACTATE NITRATE
GLUCOSE NITRATE
0.0
0.5
1.0
1.5
2.0
2.5
3.0
CELL DENSITY

Effect of respiration source on *nifB* expression.

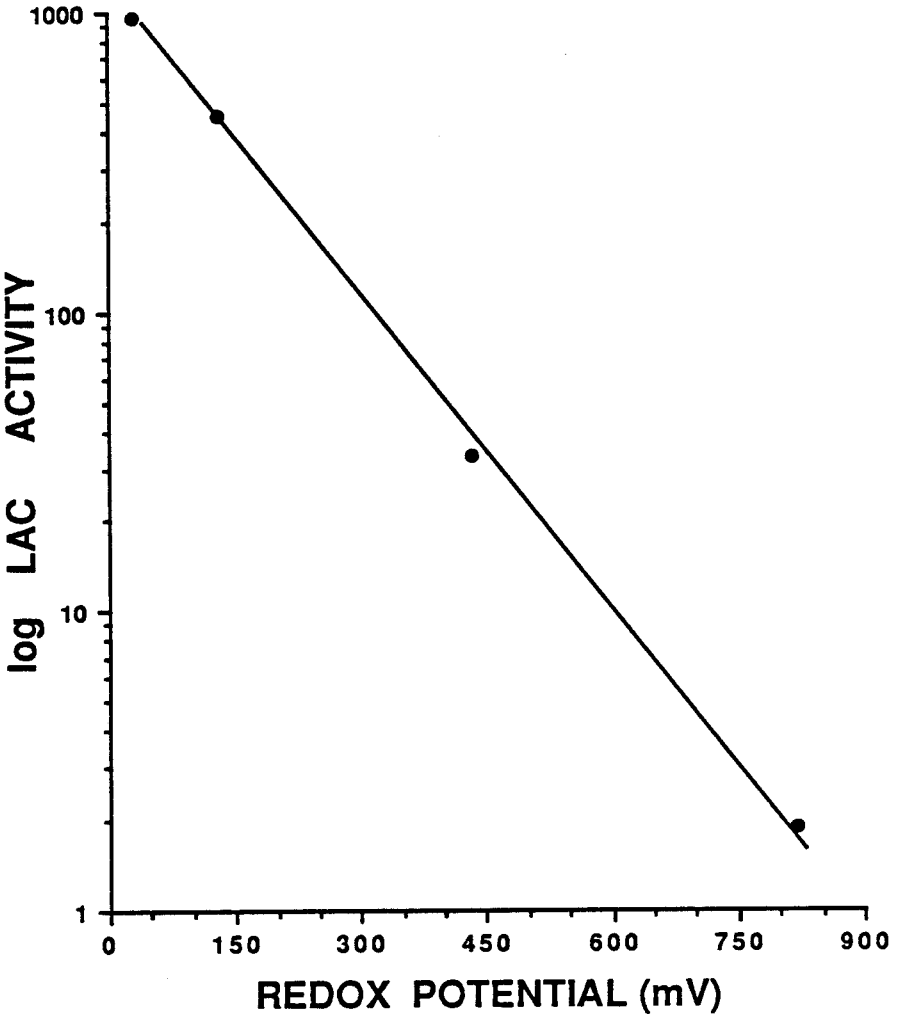
E. coli can use a variety of non-oxygen electron acceptors for anaerobic respiration (Lin 1987). Nitrate, dimethylsulfoxide, trimethylamine-N-oxide (TMANO), and fumarate will also support anaerobic growth with a non-fermentable carbon source. Each respiration source has a characteristic redox potential, and the amount of energy derived from electron transport to is determined by the difference between the redox potential of the electron donator couple, and the redox potential of electron acceptor couple (Lin 1987).

When oxygen, nitrate, TMANO, or fumarate was used as the respiration source for anaerobic growth of BZ45/pNF19 in CAM-glycerol (a non-fermentable carbon source), the final lac activity increased exponentially as the redox potential of the respiration couple decreased (Figure 11). The expression of the *nifB* promoter was exponentially related to the redox potential of all terminal electron acceptors, including oxygen. Whether the span of electron transport chain [$\Delta E_o'$] (Stryer 1981) between glycerol dehydrogenase and the terminal electron acceptor, or the extracellular redox potential (Wimpenney 1969), determines the magnitude of the respiration effect cannot be determined from this experiment. However, this result supports the conclusion that oxygen regulation of *nifB* expression depends on the redox potential of oxygen respiration, and not simple inhibition of the NifA protein by oxygen.

Figure 11

Effect of terminal electron acceptor on the expression of *nifB* during anaerobic respiration. An overnight culture of BZ45/pNF19 in CAM was diluted 1:100 in CAM-glycerol media and grown aerobicaly to an OD_{550} ~2.0. The culture was diluted 1:50 into anaerobic CAM-glycerol media with different terminal electron acceptors (oxygen, nitrate, TMANO [trimethylamine-N-oxide], or fumarate and the lac activity (Miller units) was monitored throughout the growth of the anaerobic culture. The lac activity is plotted as $\log_{10}$ of the activity determined when the culture reached stationary phase. The redox potentials are published values (Haddock and Jones 1977). Cell density is OD_{550}.

Figure 11


1000
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1
log LAC ACTIVITY
0
150
300
450
600
750
900
REDOX POTENTIAL (mV)

Control experiments for carbon source and nitrate respiration effects on *nifB* expression.

Experiments designed to elucidate a variety of points concerning carbon source and nitrate respiration effects are presented in Figure 12.

First, a mixture of the major mixed-acid fermentation products of *E. coli* (acetate, formate, and ethanol; formed in approximately equimolar ratio during mixed acid fermentation) was added to a culture prior to the start of fermentation. The final lac activity and the induction kinetics were identical to a non-supplemented xylose culture (Figure 12; induction kinetics not shown). This experiment suggests that the mixed-acid fermentation products of E. coli do not induce the continuous increase in *nifB* expression during anaerobic growth.

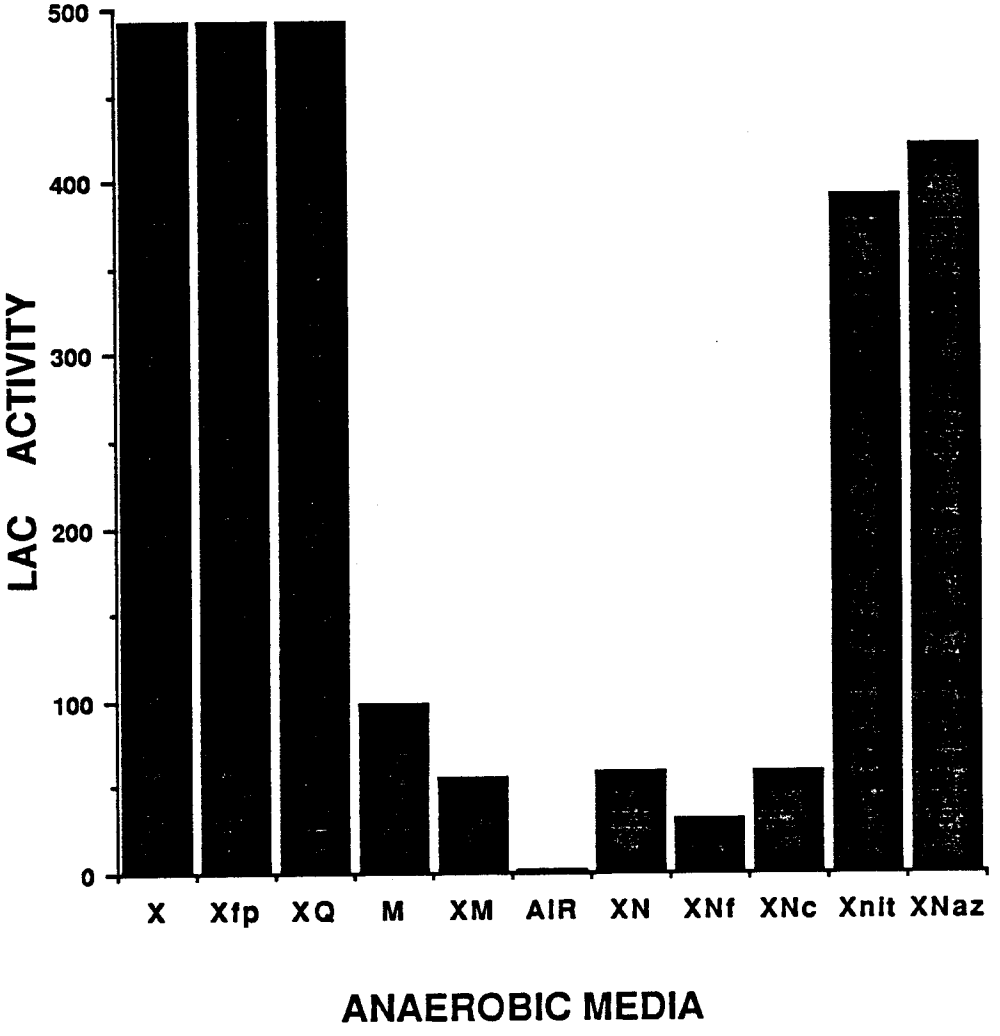
Second, fermentative growth in a mixture of xylose and maltose lowered the lac activity of the fusion (Figure 12) and the final value was attained with different kinetics (data not shown). The xylose and maltose fermentation values for the *nifB* fusion (from Figure 4) are shown for comparison.

Third, the addition of glutamine (to 10mM) had no effect on the xylose value and demonstrated that the transcription of the *nifB* promoter was not due to NtrC activation (Reitzer and Magasanik 1987) (The data in Table 2 also supports this conclusion. There is no activation of the *nifB* fusion in the absence of NifA in CAM-xylose; if CAM media was limited for glutamine and NtrC was stimulating the fusion, both the aerobic and anaerobic values would be expected to be elevated over the PE5645 control).

Figure 12

Control experiments for nifB expression. A stationary phase aerobic overnight culture of BZ45/pNF19 in CAM-xylose media was diluted 1:100 into CAM-xylose media and grown aerobically to an OD_{550} ~2.0. The late exponential phase culture was then diluted 1:50 into anaerobic CAM-xylose media supplemented with various compounds. The cultures were grown anaerobically and the lac activity (Miller units) monitored throughout the growth of the culture to stationary phase. The reported values of lac activity are those determined as the culture entered stationary phase. X, xylose; Xfp, xylose plus fermentation products (acetate, ethanol, and formate; 10 mM each); XQ, xylose plus glutamine (10mM); AIR, aerobic; XN, xylose plus nitrate (20mM, all nitrate cultures); XNf, xylose-nitrate plus formate (30 mM); XNc, xylose-nitrate plus carbonate (30 mM); Xnit, xylose plus nitrite (10 mM); XNaz, xylose-nitrate plus sodium azide (50 μM).

Figure 12


500
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0
LAC ACTIVITY
X
Xfp
XQ
M
XM
AIR
XN
XNf
XNc
Xnit
XNaz
ANAEROBIC MEDIA

Figure 12 also also shows the effect of nitrate, nitrate-formate, nitrate-carbonate, or nitrite addition to xylose media. As previously described, the addition of nitrate reduced the lac expression by ~10 fold. The addition of formate to xylose-nitrate media (formate dehydrogenase donates electrons to nitrate reductase) decreased the value still further. Formate is not a carbon source for *E. coli*, and dehydrogenation of formate produces carbonate (inorganic carbon). The addition of carbonate by itself to xylose-nitrate media had no effect. This result showed that the external addition of a substrate for nitrate reductase will increase the the repressive effect of nitrate respiration (*i.e.* - lower the lac activity). Nitrite had only a small repressive effect on xylose, perhaps due to nitrite respiration through one of three nitrite reductases in *E. coli* , only one of which generates a membrane potential (Lin 1987). This result demonstrated that nitrite, the primary product of nitrate respiration, was not responsible for the 10-fold repressive effect of nitrate in xylose cultures. The addition of sodium azide, a powerful competitive inhibitor of nitrate reductase (Forget 1974) to a xylose-nitrate culture allowed the lac activity to reach almost the fermentative value. If the repressive effect of nitrate on the *nifB* fusion was exerted through "nitrate repression" mediated by the NarL nitrate-repressor protein (Stewart et al. 1988; Nohno et al. 1989), cultures grown in the presence of nitrate would be insensitive to the addition of azide. When considered together, the results in Figure 11 and Figure 12 strongly support the conclusion that the repressive effect of nitrate and oxygen is mediated by both aerobic and anaerobic respiration.

Effect of deletions on the activity of the NifA protein.

Several reports in the published literature have shown that truncations of the NifA protein may increase, decrease, or have no effect on the proteins abililty to activate transcription (Benyon *et al.* 1988; Huala and Ausubel 1989; Morett *et al.* 1988). All of the NifA deletions tested for activity were analysed with a multi-copy plasmid fusion of the *nif*H promoter of *R. meliloti* to *lacZ* in *E. coli.* The consensus of the published reports is that amino-terminal truncations of the NifA protein retain full activity, as long as the deletion does not extend beyond the amino-terminal third of the coding sequence. Carboxy terminal deletions have been re-ported to be both inactive (Benyon *et al.* 1988; Morett *et al.* 1988) and at least half active (Huala and Ausubel 1989). Therefore, the pTTQ8 series of NifA expression vectors (see Materials and Methods) constructed for this work were transformed into *E. coli* PE56 and lysogenized with λBZ45.

On Xgal plates, pNC100/BZ45 developed dark blue colony centers essentially the same as pNF19/BZ45. None of the other truncations (pC93, pN90, pN77, pN60, or pTTQ8) produced any blue color over background (BZ45). However, this series of plasmids was found to be unstable; the pNC100/BZ45 colonies repeatedly developed ampicillin resistant, white papillae. These papillae are presumably plasmids retaining the ampicillin resistance but containing deletions or mutations of the NifA coding region. Because of this instability, no quantitative analysis of lac activity was done. However, the fact remains that none of the truncations expected to stimulate *nifB* expression were found to be active with the Xgal plate assay. Whether this is due to my use of the *nifB* promoter instead of the *nif*H promoter as in

the published literature, the use of single copy lysogens of the *nifB* promoter, or to a combination of both is unknown. It is reasonable to conclude, however, that the reported ability of NifA truncations to activate transcription may not apply to all *nif* promoters and may be an artifact of plasmid-borne fusions.

RESULTS B. The nifA promoter

Construction of the *nifA* promoter fusion.

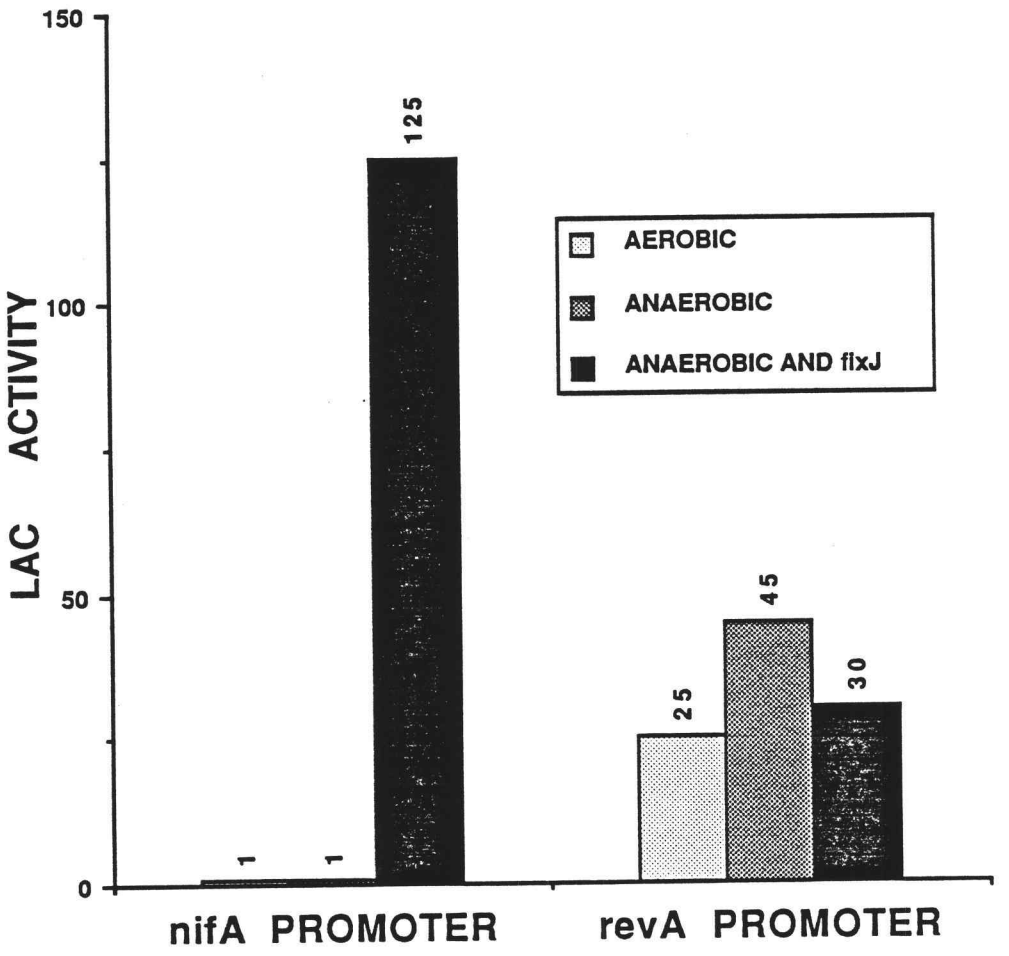
The construction of the *nifA* promoter fusion yielded two separate clones. The DNA inserted to pRS550 was capable of ligation in opposite orientations; both orientations were isolated and recombined with the lambda phage vector, generating λAZ45 and λRAZ45. These phage were then used to lysogenize *E. coli* PE56 and PE56 containing various plasmids.The initial analysis of of the two resulting strains was quantiation of the *lac* activity during anaerobic xylose fermentation. The results are presented in Figure 13 (see also Table 2).

In the absence of plasmid pCH2, (a FixJ expression vector and the gracious gift of Dr. D. Kahn, Institute Pasteur), aerobic cultures of AZ45 had 1 unit of activity (Figure 13). The addition of pCH2 had no effect when cultures were maintained in exponential aerobic growth for at least five generations at low cell densities, but overnight stationary phase aerobic cultures had 25 units of activity (data not shown). When grown anaerobically with xylose fermentation, the stimulation by FixJ was 125-fold. In contrast, the same DNA inserted in the opposite orientation had 25 units of activity in aerobic cultures, with or without pCH2, and this value increased less than 2-fold during anaerobic fermentation. pCH2 reduced the anaerobic expression by ~30%.

Figure 13

Analysis of the aerobic and anerobic expression of two oppositely oriented clones of the nifA promoter region. Overnight stationary phase aerobic cultures (AZ45, AZ45/pCH2, RAZ45, and RAZ45/pCH2) in CAM-xylose were diluted 1:100 into CAM xylose and maintained in exponential aerobic growth for at least five generations at low cell densities ($OD_{550} < 0.2$) by serial 1:5 dilutions into pre-warmed media, then collected at low cell density and the lac activity (Miller units) determined (see Material and Methods for details of collection procedure). Identical exponential aerobic cultures were made anaerobic by the addtion of Oxyrase and grown anaerobically to late exponential phase. The *lac* activity was monitored throught the growth of the anaerobic culture; the reported lac activity is that found when culture entered stationary phase. Cell density is OD_{550}.

Figure 13


150
100
50
0
LAC ACTIVITY
1
1
125
25
45
30
AEROBIC
ANAEROBIC
ANAEROBIC AND fixJ
nifA PROMOTER
revA PROMOTER

The analysis of lac activity from the *nifA* promoter fusion was complicated by similar factors that influenced the analysis of the *nifB* promoter. These were: carbon source variation, conditions for pre-growth of the innoculum, slow induction and/or transient induction of activity after shift from aerobic growth. These effects are described below.

Effect of carbon source on *nifA* expression.

When AZ45/pCH2 was grown anaerobically in different carbon sources, the lac activity was found to vary 13-fold (xylose versus glucose) (Figure 14). Maltose fermentation, which produces internal glucose and glucose-1-phosphate, yielded essentially the same activity as glucose fermentation. Compared to aerobic growth, there was a 125-fold difference in lac activity between anaerobic xylose fermentation and aerobic growth in any carbon source. The carbon source effect was undetectable in aerobic cultures (data not shown). Cultures grown fermentatively in mannitol or pyruvate had *lac* activities between that of glucose and xylose.

The addition of nitrate to each carbon source repressed lac activity (Figure 14). The repressive effect of nitrate was carbon source dependent; the lac activity in glucose-nitrate was 50% of glucose alone, while activity in xylose-nitrate was about 20% of the activity in xylose. No fermentative value is shown for glycerol (it is not-fermentable by *E. coli*) but the nitrate-linked respiration of glycerol gave higher lac activity than the nitrate-linked respiration of fermentable carbon sources.

Figure 14

Effect of carbon source and nitrate on *nifA* promoter expression. An overnight stationary phase aerobic overnight culture of AZ45/pCH2 in CAM was diluted 1:100 into CAM and grown aerobically to an OD_{550} ~2.0. The late exponential phase culture was then diluted 1:50 into anaerobic media with different carbon sources (FERMENTATION), or in the same media supplemented with nitrate (NITRATE RESPIRATION) and grown anaerobically to late exponential phase. The lac activity (Miller units) was monitored throughout the growth of the culture; the value reported is that found when the culture entered stationary phase. AIR = aerobic control culture.

Figure 14

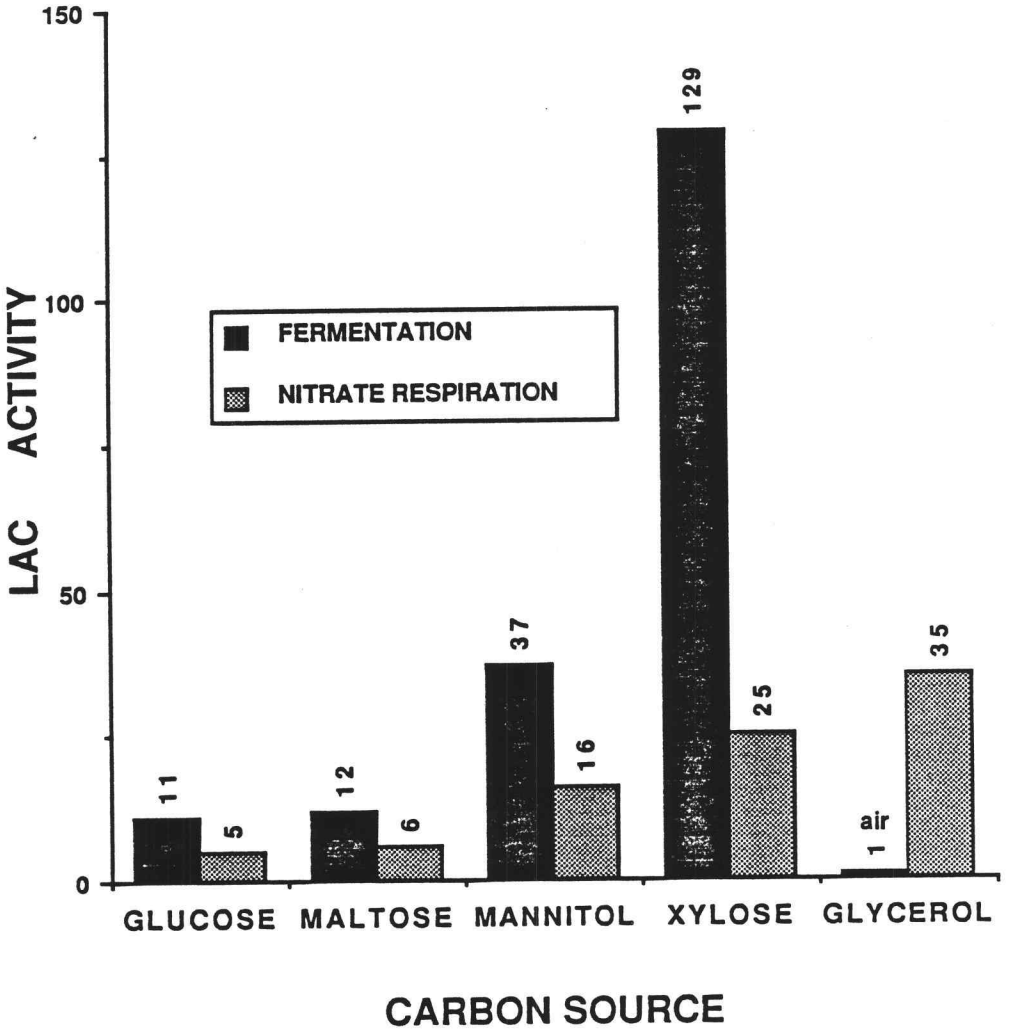
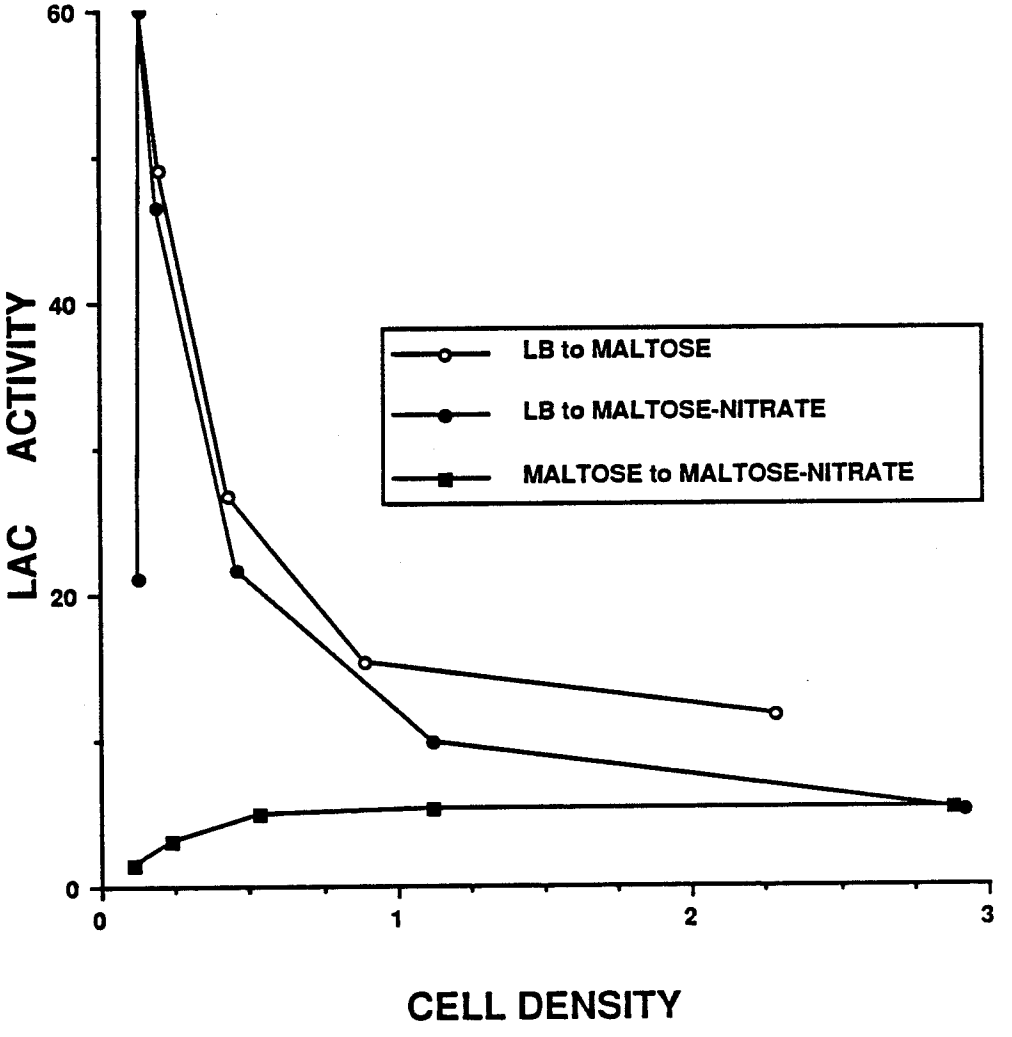

150
100
50
0
LAC ACTIVITY
FERMENTATION
NITRATE RESPIRATION
11
5
12
6
37
16
129
25
1
air
35
GLUCOSE
MALTOSE
MANNITOL
XYLOSE
GLYCEROL
CARBON SOURCE

Figure 15

Inoculum effects on anaerobic induction of *nifA* promoter activity. An overnight stationary phase culture of AZ45/pCH2 grown in Luria broth (LB) was diluted 1:50 into anaerobic CAM-maltose (LB to MALTOSE) or CAM-maltose-nitrate media (LB to MALTOSE-NITRATE) and the lac activity (Miller units) was monitored throughout the anaerobic growth of the culture. The same overnight culture was diluted 1:100 into CAM-maltose media and maintained in exponential aerobic growth for at least five generations at low cell density ($OD_{550} < 0.2$). After dilution to OD_{550} ~0.05, nitrate was added and the culture made anaerobic by the addition of Oxyrase (MALTOSE to MALTOSE-NITRATE). The lac activity was monitored throughout the growth of the culture. Cell density is OD_{550}.

Figure 15


60
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LAC ACTIVITY
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2
3
CELL DENSITY
LB to MALTOSE
LB to MALTOSE-NITRATE
MALTOSE to MALTOSE-NITRATE

Transient induction of *nifA* expression.

When cultures of AZ45/pCH2 were grown overnight in maltose or maltose-nitrate media, and then diluted 1:100 into anaerobic maltose or maltose-nitrate media, the activity was found to transiently induce by a factor of three (Figure 15). This induction occurred within the 15 minutes of transfer (no earlier times were tested), during which time the cells did not exhibit any growth (as measured by OD_{550}). When a maltose culture was maintained in exponential aerobic growth for at least five generations at low cell densities ($OD_{550} < 0.2$) and then made anaerobic by the addition of Oxyrase, the activity increases slowly to the same final value as found during the decay of transient induction (Figure 15). No decay of activity is observed when a stationary phase culture was diluted into xylose media; the culture continued to induce to the full anaerobic level of activity (see Figure 17). When a stationary phase culture was diluted to glucose or mannitol media, the rapid induction is followed by decay of the activity, as occurs with maltose (data not shown). Transient induction was also found to occur in glycerol-nitrate media, even though the aerobic culture was growing exponentially in glycerol media (see Figure 16).

Induction kinetics for anaerobic respiration.

When cultures of AZ45/pCH2 were maintained in exponential aerobic growth at low cell densities ($OD_{550} < 0.2$) in CAM-glycerol-nitrate, then made anaerobic by the addition of Oxyrase, a transient induction of activity occured (Figure 16), in contrast to maltose cultures maintained in exponential aerobic growth and then shifted to anaerobic conditions (Figure

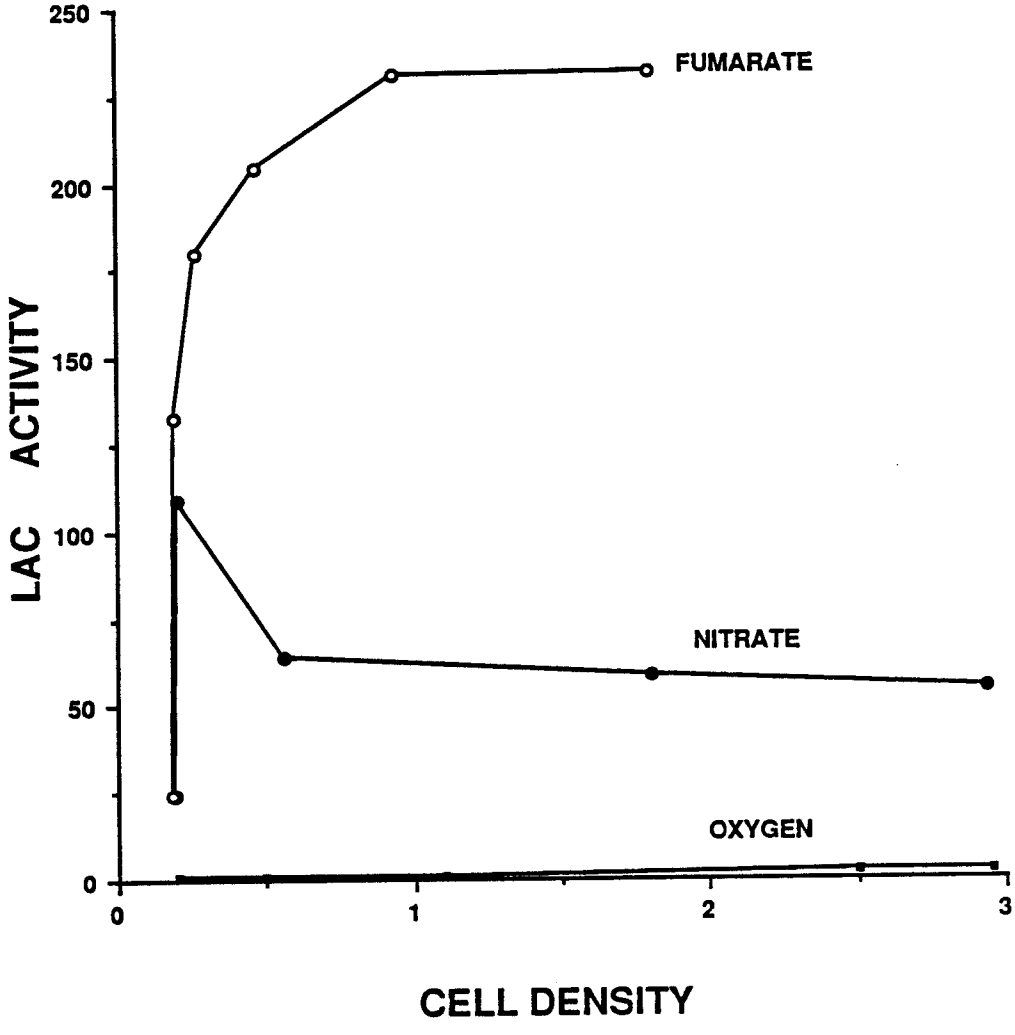
15). In this experiment, the inital measurement of lac activity was made five minutes after the shift to anaerobiosis. The transient induction was followed by a more rapid decline to the final stable value, again in contrast to maltose or maltose-nitrate media (Figure 15), in which the decay of activity occured throughout the growth phase. Glycerol is a non-fermentable carbon source, in contrast to maltose, and the transient induction of activity in glycerol may depend on the expression of anaerobic glycerol-phosphate dehydrogenase (Lin 1987). If so, the active metabolism of glycerol would be interrupted during the shift from aerobic to anaerobic conditions, but maltose catabolism should continue unimpeded (Figure 15).

Figure 16 also shows the induction kinetics for AZ45/pCH2 when an exponentially growing aerobic culture in CAM-glycerol-fumarate media is shifted to anaerobic conditions. A rapid initial induction occurs, followed by a slower, but continuous increase in lac activity. The final activity is not achieved until just before stationary phase. This result indicates that, in addition to other factors influencing transient induction, the redox potential of the terminal electron acceptor may determine whether lac activity decays after the rapid induction. Clearly, the transient induction phenomena is complex and must be considered when analyzing the induction of *nifA* promoter activity. Finally, the expression of the *nifA* promoter fusion during exponential aerobic growth is shown (Figure 16). At least until the culture begins to enter stationary phase (OD_{550} > 2.0, growth curve not shown), no expression above background was observed.

Figure 16

Kinetics of *nifA* promoter induction during respiration. A aerobic stationary phase overnight culture of AZ45/pCH2 grown in CAM was diluted 1:100 into CAM-glycerol and grown aerobically to an OD_{550} ~2.0. The culture was then diluted 1:50 into anaerobic CAM-glycerol-nitrate (NITRATE) and CAM-glycerol-fumarate (FUMARATE) and grown to late exponential phase. The lac activity (Miller units) was determined throughout the growth of the cultures. A third culture was prepared by 1:100 dilution of the overnight culture and maintained in exponential aerobic growth for at least five generations at low cell densities (OD_{550} < 0.2) by serial 1:5 dilutions in pre-warmed media. The final diluted culture was divided into five 20 mL samples in 500 mL baffled flasks with Morton closures and grown aerobically with vigorous shaking (>300 rpm) in a water-bath. One culture was collected for each lac activity determination (OXYGEN; see Materials and Methods for collection details); this procedure insures a constant culture volume/flask volume ratio for each sample as the cell density increases. Cell denstiy is OD_{550}.

Figure 16


250
200
150
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50
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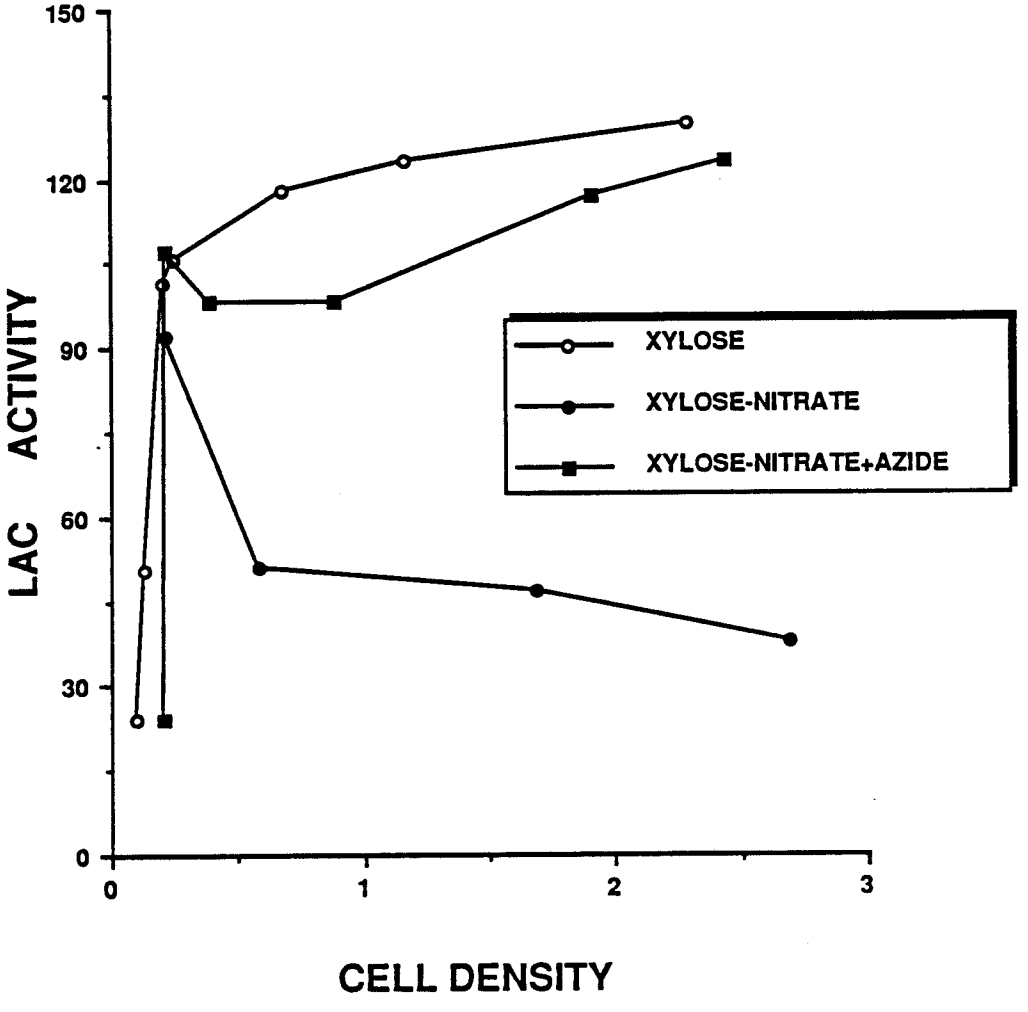
LAC ACTIVITY
FUMARATE
NITRATE
OXYGEN
0
1
2
3
CELL DENSITY

Figure 17

Inhibition of *nifA* promoter expression by nitrate respiration. An overnight stationary phase aerobic culture of AZ45/pCH2 in CAM was diluted 1:100 into CAM-xylose media and grown aerobically to OD_{550} ~2.0. The late exponential phase culture was diluted 1:50 into anaerobic CAM-xylose (XYLOSE), CAM-xylose-nitrate (XYLOSE-NITRATE), and CAM-xylose-nitrate plus 50mM sodium azide (XYLOSE-NITRATE + AZIDE) and grown to stationary phase. The lac activity (Miller units) was monitored throughout the growth of the culture. Cell density is OD_{550}.

Figure 17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LAC ACTIVITY
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CELL DENSITY
XYLOSE
XYLOSE-NITRATE
XYLOSE-NITRATE+AZIDE

Inhibition of nitrate respiration

When cultures of AZ45/pCH2 were grown overnight to stationary phase in CAM-xylose, then diluted 1:100 to anaerobic CAM-xylose media, the lac activity immediately increased 5 fold, and then increased during growth ~20% further (Figure 17). In contrast, shifting the overnight culture to CAM-xylose-nitrate media induced a transient increase in activity that decayed to a lower value, as noted for maltose. The addition of sodium azide to the xylose-nitrate culture allowed the culture to gradually reach the same value as the fermentative culture, although a small decline in the activity occured immediatedly after the rapid induction (Figure 17). The azide supplemented culture did not produce gas (NarL mediated nitrate repression was active, Lin 1987) and grew at the same rate as the fermentative culture (data not shown). Together, these results demonstrate that respiration of nitrate is required to inhibit *nifA* promoter expression.

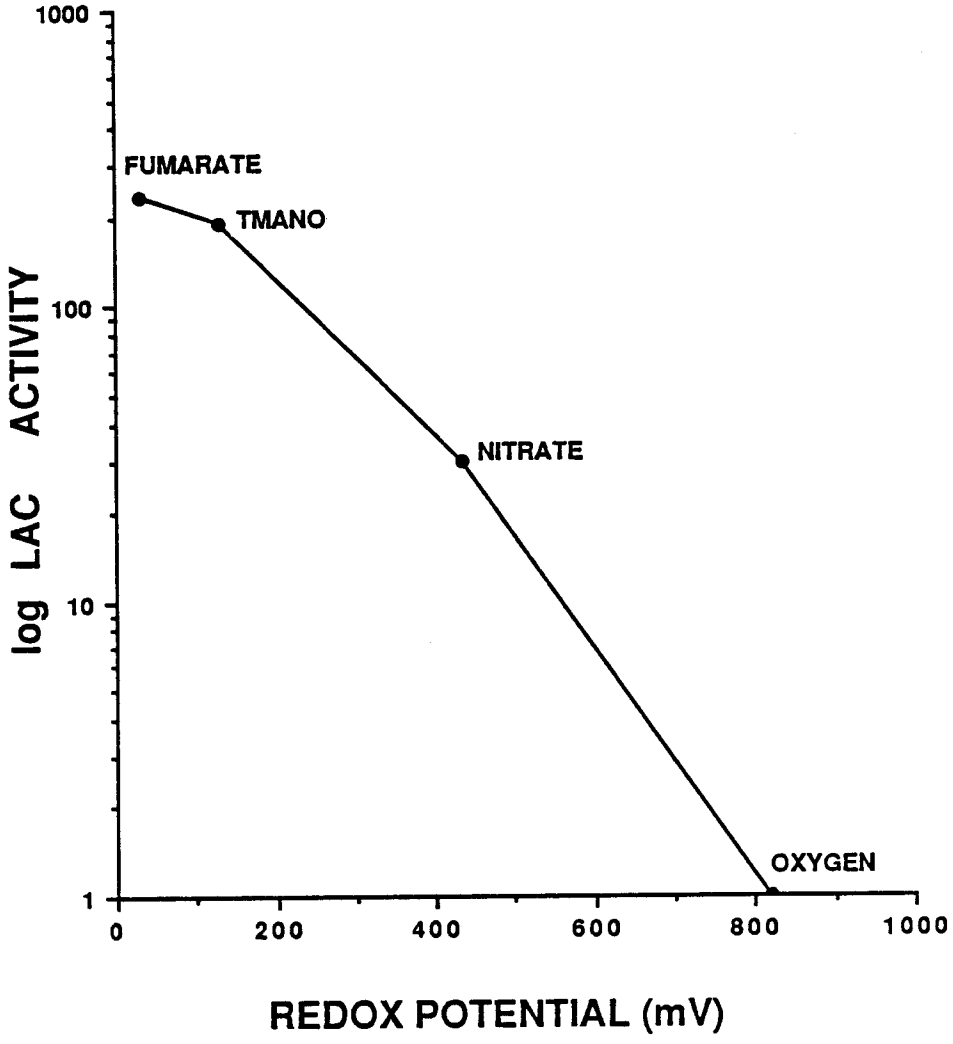
Respiration control of the *nifA* promoter.

When AZ45/pCH2 cultures were transferred from exponential aerobic growth to anaerobic CAM-glycerol media containing different terminal electron acceptors, the final anaerobic expression of the *nifA* promoter expression increased exponentially as the redox potential of the electron acceptor decreased (Figure 18). Stated in the opposite manner, the activity of the nifA promoter decreased exponentially as the redox potential of the respiration reaction increased; this effect places the reported inhibitory effect of oxygen on *nifA* promoter activity (Ditta et al. 1987) in perspective. No special effect of oxygen on the *nifA* promoter can be

Figure 18

Redox control of nifA promoter expression. An overnight stationary phase aerobic culture of AZ45/pCH2 in CAM was diluted 1:100 into CAM-glycerol and grown aerobically to OD_{550} ~2.0. The late exponential phase culture was diluted 1:50 into anaerobic CAM-glycerol media supplemented with different terminal electron acceptors for respiration (NITRATE, TMANO, or FUMARATE) and grown to stationary phase. The lac activity (Miller units) was monitored throughout the growth of the culture; the lac activity reported is when the culture entered stationary phase. A low cell density aerobic culture was also grown, as described for Figure 16, and the lac activity determined. Redox potentials are published values (Haddock and Jones 1977).

Figure 18


1000
100
10
1
log LAC ACTIVITY
FUMARATE
TMANO
NITRATE
OXYGEN
0
200
400
600
800
1000
REDOX POTENTIAL (mV)

demonstrated, other than that which can be attributed to the redox potential of oxygen respiration. Respiration of TMANO and nitrate is also repressive, when compared to fumarate respiration. A reasonable explanation for the redox effect is that an exponentially varying metabolite or physiological state is detected by the cell during respiration, and that information is transmitted (in an unknown manner) to the *nifA* promoter.

Activation of the *nifA-nifB*::lac fusion by FixJ.

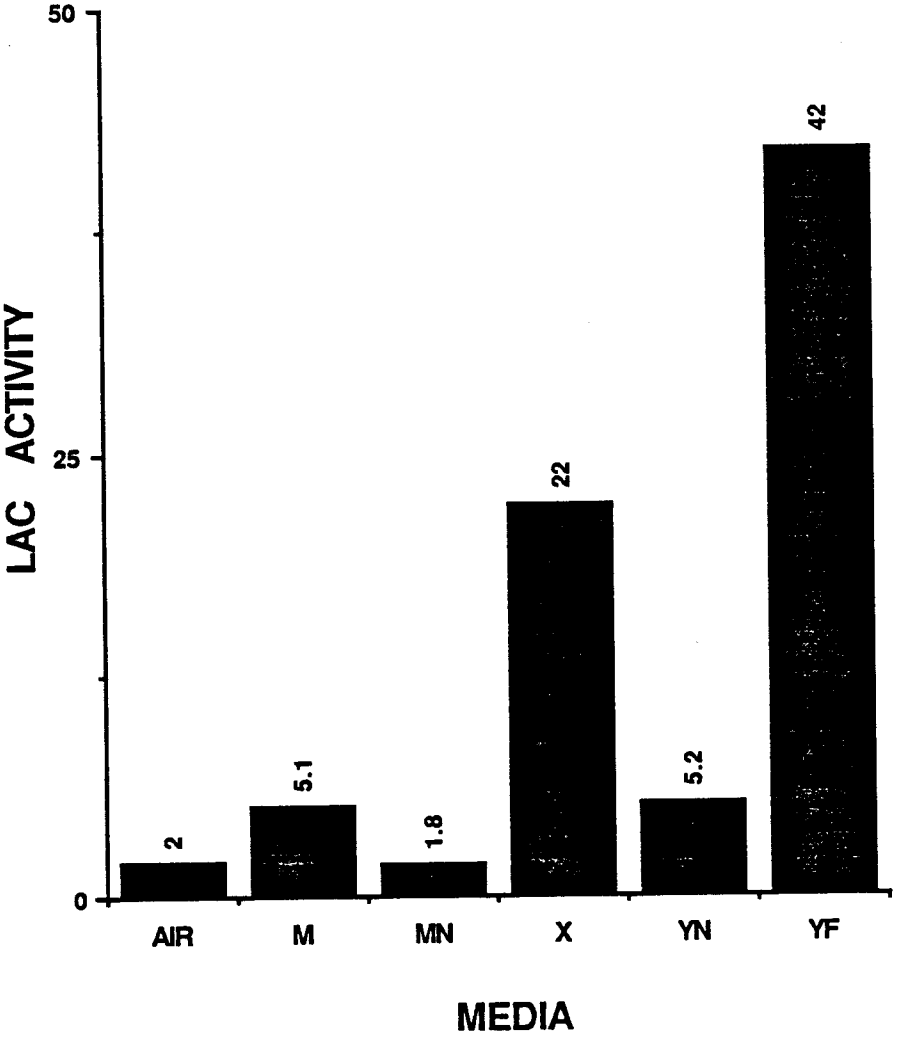
The final physiological data to be presented is for the reconstructed regulatory cascade of *Rhizobium meliloti.* By insertion of the *nifA-nifB* region of *R. meliloti* in the λRS45 phage and creating a lysogen in a strain with a FixJ expression plasmid (pCH2), the lac activity of the single-copy *nifB* promoter can be measured. In this strain (ABZ45/pCH2), the NifA protein is expressed from the pCH2 (FixJ) activated*nifA* promoter of a single chromosomally integrated *nifA* gene immediately upstream of the *nifB* gene, exactly as it occurs in *R. meliloti.*

Anaerobic cultures of ABZ45/pCH2 had very low levels of activity in anaerobic maltose media (only 2-3 fold higher than aerobic cultures) and no detectable activity over background (aerobic) in anaerobic maltose-nitrate media (Figure 19). Therefore, when NifA is produced from a single-copy of the *nifA* gene, anaerobic respiration inhibits *nifB* expression equally as well as aerobic respiration and supports the previous conclusion that respiration controls *nifB* transcription, and not oxygen.

Figure 19

Expression of the *nifB* promoter by a single copy of *nifA*. An aerobic overnight stationary phase culture of ABZ45/pCH2 in CAM was diluted 1:100 in CAM and grown aerobically to OD550 ~2.0. The late exponential phase culture was diluted 1:50 in anaerobic CAM media containing maltose (M), maltose plus nitrate (MN), xylose (X), glycerol plus nitrate (YN), or glycerol plus fumarate (YF) and grown to stationary phase. The lac activity (Miller units) was monitored throughout growth; the value reported is that found when the culture entered stationary phase.

Figure 19



The expression of the *nifB* promoter in xylose media was ~5-fold higher than in maltose, the same factor as was found for BZ45/pNF19 cultures in xylose or maltose media. This result suggests that the carbon source control of *nifB* promoter activity may be independent of the amount of NifA protein present (if the different levels of stimulation for ABZ45/pCH2 and BZ45/pNF19 is due to different amounts of NifA synthesized by the two constructs). A comparison of the expression of the *nifB* promoter when stimulated by pNF19 encoded NifA and the chromosomally encoded NifA showed that the single copy of NifA only stimulated a ~3-fold increase in expression, while the plasmid encoded NifA (Figure 4) stimulated a 50-fold increase in activity. A similar effect was observed for xylose cultures (Figure 4 versus Figure 19). Therefore, while the difference in expression between two carbon sources is similar for both sources of NifA, the level of expression is much lower when NifA is transcribed from the chromosome.

The activity of the *nifB* promoter in ABZ45/pCH2 cultures grown anaerobically in glycerol-nitrate media was essentially the same as activity in maltose media. When measured independently (strain AZ45/pCH2), the *nifA* promoter activity varied 5-fold between maltose and glycerol-nitrate media (see above). As nitrate has been shown to repress the activity of the *nifB* promoter in a similar fashion as the *nifA* promoter (see above), the effect of nitrate may be additive at the two promoters.

Cultures of ABZ45/pCH2 were stimulated ~45 fold when grown anaerobically in glycerol-fumarate media. This stimulation was ~5-fold lower than AZ45/pCH2 cultures in the same media (Figure 18) and

demonstrated that a single copy of the *nifA* gene yields low values of NifA stimulated expression with both anaerobic fermentation (maltose) and anaerobic respiration (glycerol-fumarate).

In conclusion, the stimulation of the *nifB* promoter by NifA supplied from a single copy of the *nifA* gene was reduced under all anaerobic conditions, as compared to the stimulation when NifA was constitutively expressed from a high-copy number plasmid. This effect makes it difficult to compare analysis of transcriptional activation in the published literature (in which NifA is usually supplied from a plasmid) to the data presented here.

DISCUSSION

The activation of nitrogen fixation transcriptional promoter sequences in *Rhizobium meliloti* requires the transcriptional activator protein NifA (Szeto 1984). Similarly, activation of the *nifA* gene requires the FixJ transcriptional activator protein (David *et al.* 1988) The current literature suggests that oxygen regulates transcriptional activation by NifA (Ditta *et al.* 1987; Virts *et al.* 1988) or FixJ (Batut *et al.* 1989; David *et al.* 1988). Curiously, neither by-products of oxygen respiration or unknown factors associated with oxygen metabolism seem to have been considered as factors mediating the repression of nitrogen fixation promoters. Rhizobia are (considered) obligate aerobes and the physiological analyses of NifA or FixJ gene activation in non-symbiotic cultures of *R. meliloti* can be analysed during microraerobic growth (Ditta *et al.* 1987), but not anaerobically. However, evidence for a respiration-linked dissimilatory nitrate reductase in *R. meliloti* has been presented, a curious metabolism for an obligate aerobe (Kiss *et al.* 1979).

The previous analyses of *Rhizobium meliloti* NifA activation, and the analyses presented here, were done with gene fusions. By constructing a gene fusion of a *nif* promoter to the β-galactosidase gene (*lacZ*)of *E. coli*, a sensitive enzyme assay is used to monitor gene expression. This method is particulary useful when the promoter to be analysed does not stimulate the production of an easily assayable product. For many of the *nif* genes,

including the *nifB* gene analysed here, no assay other than genetic fusion to the *lacZ* gene has been developed.

Two complementary analyses of *Rhizobium meliloti* gene regulation were chosen as the topic for this research. First, a NifA activated promoter (*nifB*) and a FixJ activated promoter (*nifA*) were fused to β-galatosidase and analysed separately. Second, the *nifA-nifB* region was fused to β-galactosidase and the interaction of FixJ stimulated *nifA* transcription and NifA-stimulated *nifB* transcription was analysed.

The *nifB* promoter was selected for analysis for two reasons: *(i)* the *nifB* promoter appeared to have a consensus NifA/NtrA promoter sequence (Buikema *et al.* 1987) but had not been rigorously shown to be a *bona fide nif* promoter, and *(ii)* the *nifB* promoter was adjacent to the *nifA* gene (less than 200 bp from the 3' end of the *nifA* gene). The location of the *nifB* promoter allowed construction of a gene fusion in which FixJ would activate a single copy of the *nifA* gene and produce wild-type NifA protein, which could then activate the single-copy chromosomally integrated *nifB* promoter. In essence, a DNA segment of *R. meliloti* encoding the regulatory cascade of *nif* gene expression was moved from the *R. meliloti* genome to the *E. coli* genome. By exploiting the well studied anaerobic metabolism of *E. coli*, the effects of oxygen on the transcription of the *nifA* promoter and on transcription directed by NifA could be analysed under defined anoxic conditions.

The analysis of gene fusions, although conceptually simple and a powerful technique for analysis of gene expression, is complicated by many

factors. Gene fusions that are designed to measure transcriptional activation often require a specific transcriptional activator protein. If the fusion is analysed in *Escherichia coli*, and the promoter under scrutiny is not homologous to an *E. coli* promoter, the activator protein must be supplied in *trans* (as is the case for the analysis of *nif* promoter activation by NifA). The complications associated with gene fusion analyses arise when both the promoter fusion and the activator protein gene reside on muti-copy plasmids.

Some of the problems with multi-copy fusion analysis are: *(i)* high background levels of expression by read-through transcription from plasmid promoters, *(ii)* titration of the activator protein by multiple copies of the promoter sequence, *(iii)* inappropriate expression of the activator protein from plasmid promoters, *(iv)* incompatibility between plasmids that limits the selection of vectors for the fusion or to supply the activating protein, *(v)* variations in the copy number of either plasmid during the growth cycle or during changes in physiology necessary for activation, and *(vi)* inherent differences in structure between plasmid DNA and chromosomal DNA (*i.e.* - topology) that may severly limit expression of plasmid-born fusions. The analysis of single-copy chromosomally located gene fusions overcomes most of these problems (Simons *et al.* 1987).

Two types of β-galactosidase fusions are commonly constructed. Fusions of a promoter to a promoterless wild-type β-galactosidase enzyme ("operon fusion"); fusions of a promoter and a portion of the coding frame of the transcribed gene in frame to a β-galactosidase gene containing an amino-terminal truncation ("protein fusion"). Fusions to the complete *lacZYA* operon of *E. coli* (when constructed in *E. coli*) allows the extensive

selection procedures developed for mutations in the lac operon to select mutants of the fused promoter (Simons *et al.* 1987). The single-copy fusion vectors designed by Simons incorporate the entire lac operon and have been used extensively to investigate the transcriptional control of anaerobically regulated genes in *E. coli* (Cotter and Gunsalus 1989; Grove and Gunsalus 1987; Sawyers and Bock 1988; 1989).

Operon fusions to the *lacZYA* operon of *E. coli* were specifically chosen for these experiments. The construction of operon fusions overcomes a potential problem with the fusion of protein coding sequences to the β-galactosidase enzyme. Although the β-galactosidase enzyme of *E. coli* retains enyme activity when many different proteins are fused to its amino-terminal, I know of no report that has determined if the *in vivo* activity of β-galactosidase enzyme with amino-terminal fusions is the same as the wild-type enzyme, and if the stability (half-life of protein decay) is the same for wild-type and "fused" enzymes. Conceivably, different enzyme activity or stability can alter the observed activity of "protein fusions". The stability and the effect of fusing NifA or NifB to β-galactosidase is unknown, and so operon fusions were chosen to measure the activity of the *nifB* (and *nifA*) promoters.

In the few instances in which multi-copy lac fusions of *nif* genes were compared to single copy fusions, large differences in expression have been found. The upstream binding site for NifA protein in the *Klebsiella pneumoniae nifH* promoter was originally described as position-insensitive, *i.e.*- the sequence could be displaced as much as 2 Kb from the wild type site and still stimulate transcription when located on high-copy number

plasmids (Buck *et al.* 1986). The same authors later showed that movement of the binding site on a low-copy number plasmid (1-2 per cell) greatly diminished expression of the fusion (Buck *et al* 1987). In *Bradyrhizobium japonicum*, multi-copy fusions of *nifD:lac* are stimulated 36-fold by NifA, while single-copy fusions are stimulated 640-fold (cited in: Gubler and Hennecke 1988). Similarly, the *fixA* and *fixBC* promoters of *B. japonicum* can only be stimulated when located on the chromosome (Gubler 1989). In *B. japonicum*, NifA is transcribed in an operon with the *fixR* gene; an intial analysis detected no auto-activation of the *fixRnifA* operon by NifA when measured with a multi-copy lac fusion (Thony *et al.* 1987). In contrast, when measured as single-copy chromosomal fusions, NifA auto-activates its own transcription by a large degree (Thony *et al.* 1989).

The analysis of *Rhizobium meliloti* nitrogen fixation promoters in *E. coli* has routinely been performed with multi-copy plasmid fusions (references cited in Literature review). The activation of single-copy *nif::lac* fusions of *R. meliloti* genes in *E. coli* has not been described. Two recent reports of *nif::lac* fusions integrated into the *R. meliloti* megaplasmid have been published (David *et al.* 1988; Batut *et al.* 1989).

Previous reports of the activation of the *R. meliloti nifB* promoter (analysed as multi-copy lac fusions) have suggested that the *nifB* promoter is much weaker than the *nifH* promoter (Morrett *et al.* 1988); another report suggests that NifB is transcribed from the *nifA* promoter upstream, and that the *nifB* "promoter" may not be physiologically relevant (Klipp *et al.*1989). Klipp *et al.* reported a 3-fold stimulation of *nifB* expression by NifA (~9 to 27 Miller units of activity) when the lac fusion was contained on a plasmid. In

contrast, I report 25 to 250-fold stimulation (depending on physiological conditions) of the *nifB* promoter when analysed as a single copy fusion. A comparison of the ABZ45, AZ45, and BZ45 fusions clearly indicates strong transcriptional termination between the *nifA* gene and the *nifB* gene, an argument against a *nifA-nifB* operon (Klipp *et al.* 1989). The original sequence analysis of the *R. meliloti nifA* gene describes a consensus termination sequence immediately after the NifA coding region (Weber *et al.* 1985), and the data presented here support that result.

A similar situation occurs with the activation of the *nifA* promoter by FixJ. The activation of the *nifA* promoter in *E. coli* by FixJ supplied from the plasmid pCH2 has been presented (Hertig *et al.* 1989). The aerobic stimulation of the *nifA* promoter was found to be 200-fold, and this value decreased by half when the cultures were grown anaerobically. This result is in contrast to the same authors analysis of FixJ activation in *R. meliloti*, in which microaerobic conditions were required for transcriptional activation by FixJ, and no aerobic expression was detected (David *et al.* 1988; see also Ditta *et al.* 1987; Virts *et al.* 1988). Using the same plasmid (pCH2), and a single-copy fusion of the *nifA* promoter to *lacZ*, I find that the promoter is not activated aerobically, and is stimulated ~200-fold (depending on physiological conditions) anaerobically, the result found in *R. meliloti* (David *et al.* 1988). This data demonstrates that single-copy fusions may be required for both activation of a nif promoter (i.e.- *B. japonicum fixA* or *B*, above), and for physiological repression of transcription.

A third difference between the results presented here and the published literature concerns the activation of *nif* promoters by truncated

derivatives of NifA. Deletion of the C-terminal region of the *R. meliloti* NifA protein has been found to reduce the transcriptional stimulation activity of NifA by 50%, when measured with a multi-copy *nifH::lac* fusion (Huala and Ausubel 1989). Deletions of the highly homologous C-terminal of *Klebsiella pneumoniae* NifA reduced activation of a multi-copy *K. pneumoniae nif*H::*lac* fusion 1000-fold (Morrett *et al.* 1988). However, for both *K. pneumoniae* and *R. meliloti* NifA proteins, deletion of the N-terminal 30% of the protein either did not effect transcriptional activation, or increased the activation (ref. cited above). Similar truncations of the NifA protein were constructed for this work, but none could stimulate expression from a single-copy *nifB* promoter fusion. Whether NifA protein truncations exhibit aberrant activation of multi-copy fusions, or the conflicting results stems from differences in the *nifH* and *nifB* promoters is not known. But, given the large differences between multi-copy and single copy fusion analysis described above, the activation of multi-copy fusions by truncated derivatives of NifA may be artifactual.

Because of the great disparity between the results found for single-copy versus multi-copy fusions, it is difficult to directly compare any of the values reported in this research to those reported in the literature. If the large disparity extends to all *nif* promoters, then the published analyses of *nif* gene regulation analysed with multi-copy lac fusions may be subject to extensive re-interpretation.

The analysis of the single-copy fusions of the *nifA* and *nifB* promoters has revealed many effects not previously described for *R. meliloti nif* promoters analysed in *Escherichia coli.* These include: carbon source

dependance of promoter activity, transient induction of activity after shifts from aerobic to anaerobic conditions, nitrate respiration-linked repression of activity that is carbon source dependant, slow induction of activity during anaerobic growth, carbon source dependance of the rate of induction during anaerobic fermentation or respiration, respiratory control of promoter activity linked to the redox potential of the respiration source, dilution-induced cessation of activity during anaerobic fermentation, and the occurence of a previously unreported promoter in the *nifA* coding region of promoter region.

With the exception of the dilution effects on expression and the α-methyl glucose inhibition of expression (which were not tested for the *nifA* promoter), all of the effects were found to occur at both the *nifA* promoter and the *nifB* promoter. It is difficult to imagine how two different promoters stimulated by two different transcriptional activator proteins could both exhibit these effects if the effects were artifactual. The effects cannot be due to the phage vector used to form the lysogenic fusions (λRS45); these effects did not occur when the same vector was used to study the transcriptional control of both anaerobic fermentation and respiration physiology of *E. coli* (Cotter and Gunsalus 1989; Grove and Gunsalus 1987; Sawyers and Bock 1988; 1989). It is entirely reasonable to conclude that the various effects are real and that the *nifA* and *nifB* promoters are regulated in a similar manner, although by different activator proteins. Whether of not the effects are physiological relevant to the symbiotic expression of nitrogen fixation genes in *R. meliloti* cannot be determined from this analysis, but two recent

reports (see below) suggest that symbiotic regulation of *nif* genes may be similar to the regulation in anaerobic *E. coli*.

Dr. David Kahn's laboratory has recently described a *lac* fusion analysis of the *fixN* and *nifA* promoters, both of which are stimulated by FixJ (Batut *et al.* 1989; David *et al.* 1988). In these analyses, a *lac* fusion was integrated into the *R. meliloti* pSym plasmid as a single-copy. For both promoters, a slow induction of activity was observed during microaerobic growth. These two results are the sole support for the effects I observed in *E. coli*. No carbon source or nitrate effects were reported, if indeed they were studied. In any case, at least one of the effects - slow induction of lac activity - does occur in micro-aerobic cultures of *R. meliloti*.

The discovery of the reverse promoter in the *nifA* gene is interesting, but it is not known if the promoter functions in *R. meliloti*. Kim has presented evidence for read-through transcription of the *nifA* gene from the upstream *fixABCX* promoter (Kim *et al.* 1986). Various reverse-orientation chloramphenicol-acetyl-transferase fusions were isolated during their analysis and no evidence for a reverse promoter was found (data not shown). They did not state which of the CAT fusions were tested for opposite strand promoter activity.

It is possible that the presumed promoter *revA* is simply a DNA sequence that *E. coli* can recognize as a promoter. If the promoter is a functional *R. meliloti* promoter, then the RNA transcript could theoretically be isolated and analysed by standard methods of RNA transcript analysis. This would not illuminate the function of the promoter, but would at least

determine if it is an *E. coli* artifact. The fusion strain RAZ45 could be used to isolate mutants of the promoter, and the mutant sequnce then re-cloned in the opposite orientation to analyse effects of the inactive promoter on FixJ stimulated expression of the *nifA* promoter. Conceivably, the *revA* promoter may be an additional source of control for the *nifA* gene.

The NifA protein has been proposed to be oxygen sensitive and experiments that suggest *Bradyrhizobium japonicum* NifA is irreversibly inactivated by oxygen have been presented (Kullik *et al.* 1989 and references cited therein). The results of Kullik *et al.* are based primarily on the following experiment: when NifA is synthesized aerobically in *Eshcherichia coli*, then the cells are killed by the addition of chloramphenicol and incubated anaerobically (chloramphenicol is added to halt any synthesis of NifA under anaerobic conditions), no accumulation of mRNA from a nitrogen fixation promoter fusion to β-galactosidase occurs. The authors concluded that NifA protein synthesized under aerobic conditions is unable to activate the promoter. However, this experiment does not address the following question: Is aerobically produced NifA truly inactive in chloramphenicol-killed cells (as they suggest), or is aerobically produced NifA inactive because chloramphenicol-killed cells are unable to shift their previous aerobic metabolism to anaerobic metabolism? Would aerobically produced NifA be active <u>if</u> the cells were able to shift to anerobic metabolism, *i.e.* - is the NifA protein inactive aerobically, or is it only inactive in the dead cells that were grown aerobically? While this may be a fine point of contention, it is critical for the analysis of NifA function. It is necessary to distinguish if the NifA <u>protein</u> is sensitive to oxygen (or by-

products of oxygen metabolism), or if the NifA protein is sensitive to a physiological state that occurs during oxygen metabolism. I contend that the difference is crucial. In the first case, post-translational modification of the protein is a controlling feature of NifA directed gene activation; in the second, the physiological state of the *Rhizobium* cell is the critical feature.

The results presented here support the conclusion that cellular metabolism regulates *Rhizobium meliloti nif* gene expression. In addition, the data support the conclusion that the aerobic repression of NifA and FixJ directed transcription is exerted by the physiological conditions present during oxygen respiration; however, the repression by oxygen is only a matter of degree, as other forms of respiration are also inhibitory.

As noted above, numerous effects were discovered during the analysis of single-copy *nifA* and *nifB* fusions that have not been previously described. With the exception of the effects of redox potential on *nif* gene expression, all effects were related to carbon catabolism. The *lac* activity varied 10-fold depending on carbon source; the degree of repression by nitrate respiration was carbon source dependent; transient induction of activity occured when shifting from aerobic to anaerobic catabolism of glycerol, but not maltose; the addition of an inhibitor of glucose catabolism (α-methyl glucose) caused a 5 to 10-fold increase in lac activity; and finally, growth on two carbon sources (xylose and maltose) decreased expression of the fusion activity.

The different levels of expression in different carbon-supplemented media is not due to the well-known effect of cyclic-AMP (cAMP) (see

Results). Carbon source dependent expression and glucose repression of anaerobically stimulated genes in *E. coli* has been observed previously (Cotter and Gunsalus 1989) and both effects have been shown to be independent of cAMP (Cotter and Gunsalus 1989; Reams and Clark 1988). It seems that there is an anaerobically regulated form of catabolite repression in *E. coli* that is not cAMP mediated. By inference, the same catabolite repression system occurs in *R. meliloti*, and *R. meliloti* nif genes are sensitive to the factor(s) in *E. coli* that mediate the repression. Non-cAMP catabolite repression occurs in *R. meliloti* and has been reviewed recently (O'Gara *et al.* 1989). An artifactual control by the *E. coli* anaerobic, non-cAMP catabolite repression system seems very unlikely.

The transient induction effect and the effect of α-methyl glucose can be reconciled as two different aspects of the same carbon metabolism control. If the effect of carbon source is exerted through the rate of carbon catabolism, or the production of some compound whose equilibrium concentration in the cell is related to the carbon catabolism, then changes in the rate of carbon catabolism should effect the expression of fusion activity.

When cultures of BZ45/pNF19 were shifted from exponential aerobic growth in maltose to anaerobic maltose fermentation (Figure 6) or maltose-nitrate respiration (data not shown), the induction of expression occurred smoothly, with a transient increase in activity. Conversely, exponentially growing cultures in glycerol media underwent an immediate transient induction of activity when shifted to anaerobic conditions. Anaerobic glycerol utilization requires the induction of a specific anaerobic glycerol-3-

phosphate dehydrogenase (Lin 1987), while maltose catabolism continues with the same enzymes used aerobically. The transient induction is proposed to occur when the catabolism of glycerol is interrupted during the shift from aerobic to anaerobic conditions. The results of α-methyl glucose addition to fermentative glucose cultures supports this hypothesis.

α-methyl glucoside is a substrate of the glucose PTS system, the assembly of proteins which internalize glucose with its concomittant phosphorylation (De Reuse and Danchin 1988; Grimont and Bouvet 1989). The methyl group prevents further catabolism of the phosphorylated α-methyl glucose; α-methyl glucose is therefore a competitive inhibitor of glucose uptake (the kinetics of uptake are essentially identical to glucose), while at the same time a source of wasted energy for any glucose that does enter (α-MG consumes a single molecule of the glycolytic intermediate phosphoenolpyruvate to phosphorylate the sugar). Therefore, the addition of α-MG in equal concentration to glucose will halve the rate of glucose consumption through simple mass-action, and waste half of the high-energy phosophate intermediates generated by glycolysis (disregarding any contribution of the pyruvate-formate lyase/acetate kinase pathway for ATP generation). As shown in Figure 9, a 50% change in the rate of carbon consumption causes a 6-fold increase in the lac activity, while a nine fold difference between glucose and α-methyl glucose increases the lac activity only ~25% more than a 1:1 mixture of glucose and α-methyl glucose.

The addition of xylose to a fermentative maltose culture of the *nifB* fusion strain was shown to reduce the expression of the promoter. This effect can also be explained by the hypothesis discussed above. By adding

a second carbon source that (presumably) increases the net rate of carbon catabolized by the cell, the activity of the fusion is depressed. This results suggest that it is not a specific catabolite formed during fermentative glycolysis that acts as an inhibitor, but some factor associated with the net carbon catabolism (or net rate of carbon catabolism). Similarly, it is important to note that the addition of α-methyl glucose did not change the carbon source that the cells could use for glycolysis, but only the rate at which the carbon (glucose) could be catabolized.

The carbon source dependent repression of activity by nitrate respiration may also be considered as one facet of the more general system of carbon catabolism control. There is a 10-fold difference in the activity of the *nifB* promoter in glucose-nitrate versus xylose-nitrate media (Figure 4). But, while the activity in xylose-nitrate media is 5-fold higher than in glucose-nitrate media (50 units vrs. 10 units; Figure 4), the degree of repression by nitrate is almost twice as much for xylose fermentation as for glucose fermentation (10-fold repression by nitrate in xylose; 6-fold for glucose). In other words: if the fermentative activity increases from one carbon source to another, so does the nitrate respiration activity; but the degree of repression by nitrate is proportionally higher also. How can this result be integrated with the other effects of carbon catabolism?

A reasonable explanation is that the energy derived from the catabolism of any carbon source also regulates the expression of the *nifA* or *nifB* promoter. The effect of α-methyl glucose supports this hypothesis; α-MG not only limits glucose entry, but in essence "wastes" some of the high-energy phosphates generated by glycolysis. The addition of nitrate, and

thereby the ability to generate energy without "wasting " carbon by the excretion of mixed acid fermentation products, could increase the internal pools of gycolytic products available for anabolic reactions and/or increase the net rate of carbon catabolism. In effect, the addition of nitrate to xylose media may have a larger effect on the cells ability to generate energy, than it does for a rapidly metabolized carbon source such as glucose (xylose must make a circuitous trip through the pentose-phosphate pathway before entering glycolysis at fructose-6-phosphate and does not support rapid growth aerobically or anaerobically).

The *nifB* promoter activity is at least 10-fold higher in lactate-nitrate media than in glycerol-nitrate media, and this result is consistent with the hypothesis that both carbon catabolism and the energy generated by the carbon catabolism influence the expression of the *nifB* promoter. Lactate and glycerol are non-fermentable by *E. coli*, and both the anaerobic glycerol-3-phosphate and lactate dehydrogenase enzymes will donate electrons and hydrogen to nitrate reductase (Lin 1987; Stewart 1988). However, glycerol enters the glycolytic pathway at glyceraldehyde-3-phosphate and generates NADH and ATP during its conversion to pyruvate. The NADH serves as an additional electron donor to nitrate reductase (Lin 1987). Lactate is converted to pyruvate by the dehydrogenase without production of NADH or ATP. Indeed, pyruvate consumes two high energy phosphates to make PEP and therefore enters glycolysis "at the bottom" and with an energy debit that cannot be repaid by any substrate-level generation of ATP. In summary, the anaerobic catabolism of two different non-fermentable carbon sources leads to two very different levels of

expression, and the expression can be reconciled with the differences in the catabolism of the two compounds.

The effect of redox potential on expression seems to be separate from the effect of carbon catabolism. A recent analysis of the anaerobic control of cobinamide biosynthesis in *Salmonella typhimurium* (Andersson and Roth 1989; Escalante-Semerena and Roth 1987) has shown a very similar pattern of redox control as that found for the *nifA* and *nifB* promoters. However, for the cobinamide system, the lac activity of of single-copy fusions was found to decrease linearly with the increasing redox potential, while the *nif* promoters exhibit a logarithmic decrease in expression as the redox potential of the terminal electron acceptor increases.

The exponential decrease in promoter activity explains why oxygen has been considered as a specific inhibitor or *nifA* promoter expression and NifA-directed stimulation of *nif* promoter expression. But, the published literature has uniformly compared the promoter activity during oxygen respiration to anaerobic fermentation; in this work, oxygen respiration has been compared to anaerobic respiration of other compounds. When the two types of respiration were compared in defined media with a non-fermentable carbon source (to preclude any effects of substrate-level phosphorylation as a source of energy), no effect of oxygen was found that could not be accounted for by the redox potential of oxygen respiration. It is the exponential effect of redox potential that makes oxygen <u>appear</u> as a special inhibitor of *nif* gene expression, but only when it is compared to non-respiratory anaerobic fermentation. In *Klebsiella pneumoniae,* nitrate <u>respiration</u> has been shown to inhibit *nif* gene expression (Hom *et al.* 1980,

Pecher *et al.* 1983), as I have described here for *R. meliloti* gene fusions in *E. coli.*

What factors could account for the exponentially decreasing expression of the *nifA* and *nifB* promoters as the redox potential of the terminal electron acceptor increases? A likely candidate could be an equilibrium constant for two different redox states of a metabolite, *i.e.* oxidized versus reduced. Redox potential is directly related to the free energy of the redox reaction through the Nernst equation, and the free energy of a reaction can be expressed as the logarithm of a equilibrium constant (Stryer 1981). Therefore, the exponential increase in lac activity as the redox potential of the terminal electron acceptor becomes smaller may be determined by the redox state of a metabolite, or to some other exponentially varying state that occurs under anaerobic conditions.

One metabolite that varies by many orders of magnitude between aerobic and anaerobic conditions is the concentration of quinone intermediates (reviewed in Stewart 1988; and Lin 1987). The ratio of ubiquinone to menaquinoe is ~20 in aerobic cultures of *E. coli*, while menaquinoe is the only detectable quinone in anaerobic fermentative cultures, or anaerobic cultures respiring fumarate and with a non-fermentable carbon source. Anaerobic nitrate-respiring cultures have a depressed, but measureable content of ubiquinone. How the redox state of the quinone molecule is sensed, or how the ratio of different quinones contributes to the redox-sensing system of the cell is unknown. It is known that the transcription of ubiquinone synthetic enzymes is regulated by the ArcA repressor (Iuchi and Lin 1988). Similarly, how the redox potential of the

terminal electron acceptor is sensed and the information translated into different levels of expression of the *nifB* promoter is unknown and will require further investigation.

Finally, what accounts for the slow increase in *nifB* and *nifA* promoter activity? The results of the anaerobic dilution experiments suggests that dilution of some excreted metabolite could account for the transient (but complete) repression of promoter activity. Interestingly, a variety of *E. coli* proteins have been found to increase their concentration slowly after a shift from aerobic to anaerobic growth (Smith and Neidhardt 1983); the authors also suggest that the concentration of excreted metabolite may account for the slow increase in the cellular concentration of the anaerobically induced proteins. The slowly induced proteins were all repressed by the addition of nitrate to the anaerobic cultures, a further (perhaps not coincidental) similarity with the expression of the *nif* promoters described in this work.

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